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(54) Title: A FUNGUNS WHEREIN THE areA, pepC AND/OR pepE GENES HAVE BEEN INACTIVATED

(57) Abstract

The present invention relates to fungi, which do not produce proteases. The fungi of the invention are useful as hosts for the production of proteins susceptible of proteolytic degradation by the proteases usually produced, and the invention consequently encompasses processes for the production of proteins of interest in high yields by using the fungi of the invention. The invention also comprises methods for producing such fungi and DNA constructs to be used in these methods.

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Title: A fungus wherein the are A, pep C and/or pep E genes have been inactivated

FIELD OF THE INVENTION

The present invention relates to fungi, which do not produce proteases. The fungi of the invention are useful as hosts for the production of proteins susceptible to proteolytic degradation by the proteases usually produced, and the invention consequently encompasses processes for the production of proteins of interest in high yields by using the fungi of the invention. The invention also comprises methods for producing such fungi and DNA constructs to be used in these methods.

BACKGROUND OF THE INVENTION

Fungi, and especially filamentous fungi, are widely used commercially because of their ability to secrete remarkably high levels of proteins

Among the filamentous fungi species belonging to the genus *Aspergillus* have a long history of commercial use for the production of endogenous and lately also heterologous proteins.

One disadvantage with most microorganisms used for the production of proteins is the inherent production of proteases which may subject a protein product of interest to degradation due to proteolysis.

Various ways of avoiding this have been envisaged. Among other solutions it has been suggested to delete or disrupt the genes encoding the various proteases. Unfortunately, the fungi produce a high number of proteases making such a solution more or less unrealistic.

A need is therefore persisting for strains of filamentous fungi exhibiting no or very low levels of protease production.

For a number of years it has been known that the regulatory gene *areA* which mediates nitrogen metabolite repression in *A. nidulans* influences the production of

extracellular proteases (Arst & Cove, *molec. gen. Genet.* **126**, (1973) 111-141).

The *areA* gene from *A. nidulans* has been cloned (Caddick *et al.*, *EMBO Journal* **5**, (1986) 1087-1090) and various modifications made to it to evaluate functions of different regions in the activator protein encoded by this gene (Stankovitch *et al. Mol. Microbiol.* 7, (1993) 81-87). Furthermore the gene coding the corresponding function in *A. fumigatus* apparently has been cloned recently (Hensel *et al.* 2nd European Conference on Fungal Genetics, April 28 to May 1, 1994, Book of Abstracts, E11).

From the literature a single use is also known of a strain of *A. nidulans* of genotype *argB areA1* as a host for the production of t-PA (Upshall *et al. Biotechnology* 5, (1987) 1301-1304). In this example only the *argB* genotype is used as a selection marker through its arginine prototrophy, while the *areA* genotype is simply a coincidence.

International Patent Publication No. WO 95/35385 discloses the deletion of the *areA* gene as a means for reducing the protease level in filamentous fungi.

Apart from the extracellular proteases, fungi also produce a number of intracellular proteases (also called endoplasmic).

Among these a serine protease of the subtilisin type produced by *A. niger* and designated PepC has been described, the gene expressing it cloned, and a deletion mutant described in EP 574 347 and in Frederick *et al.*, *Gene*, **125** 57-64 (1993)

A further such protease of the aspartic type designated PepE has been disclosed in Jarai *et al.*, *Gene*, **145** 171-178 (1994). the article discloses the cloning and characterisation of the *pepE* gene and speculates about the regulation of the *pepE* and *pepC* genes.

The present invention has as an object the alleviation of the need for protease free filamentous fungi.

SUMMARY OF THE INVENTION

The present invention consequently relates to fungi, wherein the *areA*, *pepC*, and/or *pepE* genes by recombinant DNA technology have been modified such that they cannot be expressed in a way providing for a functional AreA activator and functional PepC and/or PepE proteases.

The invention furthermore relates to methods for producing such fungi, obtained by deletion of the *areA*, *pepC*, and/or *pepE* genes.

This may be obtained through a method comprising

i) cloning of the *areA*, *pepC*, and/or *pepE* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *areA* gene, the *pepC* gene, and the *pepE* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *areA*$^-$, *pepC*$^-$, and/or *pepE*$^-$.

The information obtained from the above mentioned cloning of the *areA*, *pepC*, and/or *pepE* genes may also be used in connection with the well-known anti-sense technology, to construct an expression plasmid giving rise to synthesis of a RNA molecules complementary to the mRNA transcribed from the *areA*, *pepC*, and/or *pepE* genes, and to transform the fungus of interest therewith.

The invention furthermore relates to DNA constructs intended for use in the above mentioned methods.

Furthermore the invention relates to methods of producing a desired protein or gene product, especially secreted proteins, whereby a fungal host modified and optionally transformed with a DNA construct comprising at least a DNA sequence coding for the protein or gene product of interest, is

cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

When working with the invention it was surprisingly found that the fungi of the invention produces such secreted proteins in a much improved yield.

It was also surprisingly found that the only nitrogen source capable of providing good growth of the *A. oryzae areA* strains was glutamine.

The invention furthermore relates to protein products produced by the above methods.

Also the invention relates to a DNA sequence coding for the *pepC* gene from *A. oryzae* (SEQ ID No. 1) or functional alleles thereof.

The invention also covers a PepC protease from *A. oryzae* (SEQ ID No. 2), and processes for the production of the PepC protease comprising transforming a suitable host with a DNA construct comprising a DNA sequence coding for the PepC protease, selecting a transformant capable of producing said PepC protease, cultivating said transformant in an appropriate growth medium and recovering said PepC protease from said culture.

Furthermore the invention relates to a DNA sequence coding for the *pepE* gene from *A. oryzae* (SEQ ID No. 3) or functional alleles thereof.

Also, the invention relates to a PepE protease from *A. oryzae* (SEQ ID No. 4), and processes for the production of the PepE protease comprising transforming a suitable host with a DNA construct comprising a DNA sequence coding therefore, selecting a transformant capable of producing said PepE protease, cultivating said transformant in an appropriate growth medium and recovering said PepE protease from said culture.

According to these aspects said host is preferably a fungus, according to the invention, especially *A. oryzae*, and wherein said DNA construct provides for an extra copy of the gene encoding either said PepC or PepE protease.

BRIEF DESCRIPTION OF THE DRAWING

The invention is described in further detail in the following parts of the specification with reference to the Examples and the drawing, wherein

Fig. 1 shows the steps involved in the construction of HowB101,
Fig. 2 shows the construction of pToC345,
Fig. 3 shows the steps involved in the construction of pToC315.
Fig. 4 diagrammatically shows a two step gene deletion of the *pyrG* gene.
Fig. 5 shows the construction of pJaL235,
Fig. 6 shows the steps involved in the construction of pJaL335,
Fig. 7 shows the steps involved in the construction of pJaL363,
Fig. 8 shows the steps involved in the construction of pJaLz,
Fig. 9 shows the construction of pSK5 and pSK9,
Figs. 10a and 10b show the steps involved in the construction of pToC243 and pToC266,
Fig. 11 shows the steps involved in the construction of pMT1606,
Fig. 12 shows the construction of pToC56,
Figs. 13a and 13b show the steps involved in the construction of pJaL368, and
Figs. 14a and 14b show the construction of pToC338.

DEFINITIONS

In the present specification the following definitions are used:

The expression *areAD* means a strain in which the *areA* gene is deleted. Similar notations are used for strains, wherein the *pepC*, and/or *pepE* genes are deleted.

The expression *areA*$^-$ means a strain which does not produce a functional AreA activator. The term "loss of function"

is also often used for this. Similar notations used for strains, which do not produce functional PepC, and/or PepE protease(s).

The expression "anti-sense technology" describes methods such as disclosed in US Patent No. 5,190,931.

DETAILED DESCRIPTION OF THE INVENTION

As indicated the present invention relates in its first aspect to fungi, wherein the *areA* gene by recombinant DNA technology has been modified in a way by which it cannot be expressed in a way providing for a functional AreA activator, and wherein the genes encoding for the extracellular proteases PepC and/or PepE has been inactivated in a manner whereby they are not expressed to produce functional proteases.

This object may specifically be obtained by deletion or disruption of the *areA*, *pepC*, and/or *pepE* genes.

The cloning of the *areA*, *pepC*, and/or *pepE* genes are described in the Examples.

AreA homologs from other fungi could be cloned either by cross hybridization with one of the already known genes or by complementation of *areA* mutants; e.g. *A. nidulans areA*-18 or the *A. oryzae areA* deleted strain described in this application.

Methods for deleting or disrupting a gene are specifically described in WO 90/00192 (Genencor).

Methods for substituting DNA in a gene are also generally known, and can be accomplished by substituting one or more continuous parts of the gene, but it may also be obtained by site directed mutagenesis generating a DNA sequence encoding a AreA activator variant that is not functional.

Another method by which such an object may be obtained is by using anti-sense technology.

The anti-sense technology and how to employ it is described in detail in the aforementioned US Patent No. 5,190,931 (University of New York).

A further method of obtaining said inactivation is by inserting extra DNA internally in the *areA* gene, thereby giving rise to the expression of a dysfunctional activator protein.

In connection with this method information provided by the cloning can be used to make DNA constructs that can be integrated into the *areA* gene, and even replace it with another gene, such as the *pyrG* gene.

A further method of avoiding the presence of the *areA* activator is by interfering with the regulation of the expression signals regulating the expression of the *areA* gene itself.

The principles described above apply equally to the *pepC*, and/or *pepE* genes.

According to the invention the fungus preferably belongs to a genus selected from the group comprising *Aspergillus*, *Trichoderma*, *Humicola*, *Candida*, *Acremonium*, *Fusarium*, and *Penicillium*.

Among these genera species selected from the group comprising *A. oryzae*, *A. niger*, *A. awamori*, *A. phoenicis*, *A. japonicus*, *A. foetidus*, *A. nidulans*, *T. reesei*, *T. harzianum*, *H. insolens*, *H. lanuginosa*, *F. graminearum*, *F. solani*, *P. chrysogenum*, and others are preferred.

As indicated the invention also is meant to encompass the method for producing the fungi of the first aspect of the invention, and wherein said inactivation has been obtained by deletion of the *areA*, *pepC*, and/or *pepE* genes, which method comprises

i) cloning of the *areA*, *pepC*, and/or *pepE* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *areA* gene, the *pepC* gene, and/or the *pepE* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *areA*$^-$, *pepC*$^-$, and/or *pepE*$^-$.

Since it is believed that the maturation of the PepC protease is controlled by the PepE protease the invention also comprises a method for producing a fungus of the invention, wherein said inactivation has been obtained by deletion of the *areA* and *pepE* genes, which method comprises

i) cloning of the *areA* and *pepE* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *areA* gene and the *pepE* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *areA*$^-$, and *pepE*$^-$.

Also included is the method for producing the fungi, wherein the inactivation has been obtained by using anti-sense technology. Such a method comprising

i) construction of expression plasmids, each of which give rise to synthesis of an RNA molecule complementary to the mRNA transcribed from the *areA* gene, the *pepC* gene, and/or the *pepE* gene,

ii) transformation of the host fungus with said expression plasmids and a suitable marker, either on separate plasmids or on the same plasmid,

iii) selection of transformants using said marker, and

iv) screening selected transformants for strains exhibiting a reduction in the synthesis of the AreA, PepC, and/or PepE products.

A further aspect of the invention is meant to comprise DNA constructs for use in the above mentioned methods.

In respect of the former method said DNA constructs may comprise the *areA*, *pepC*, and/or *pepE* genes, wherein an internal part has been substituted, deleted, or extra DNA has been inserted.

At least one of the DNA constructs may furthermore also comprise DNA sequences encoding a protein product of interest, such as those mentioned later.

In respect of the latter anti-sense method the DNA constructs may comprise inverted DNA sequence of the *areA*, *pepC*, and/or *pepE* genes connected to a functional promoter, whereby the mRNAs are at least partially complementary to mRNAs produced from the *areA*, *pepC*, and/or *pepE* genes.

A further aspect of the invention relates to a process for the production of a desired gene product, preferably a secreted gene product, whereby a fungus according to the invention is cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

In the case of a gene product expressed by a heterologous gene the DNA sequence coding for the desired gene product may be a part of the DNA construct used for producing said fungus.

Normally, however, a separate transformation of the fungus of the invention is performed in order to make the fungus capable of producing the desired product.

Methods for transforming fungi are well known in the art, cf. e.g. EP 0 184 438 A2 (Gist-Brocades N.V.) and EP publication No. 0 98 993 (Novo Nordisk A/S).

For indigenous products this is of course not necessary, but in order to increase the production it may be an advantage to provide for multiple copies of the gene encoding the protein of interest to be incorporated into the host.

The desired gene product is generally a peptide or protein, preferably an enzyme.

Among enzymes it is preferably selected from the group comprising proteases, such as trypsin and chymosin; lipases, cutinases, cellulases, xylanases, laccases, pectinases, etc.

Another type of desired gene product is generally a therapeutically active peptide or protein.

Among the therapeutically active peptide or protein the protein preferably is selected from the group comprising insulin,

growth hormone, glucagon, somatostatin, interferons, PDGF, factor VII, factor VIII, urokinase, t-PA, CSF, lactoferrin, TPO etc.

A further aspect of the invention relates to the DNA sequences coding for the *pepC* gene from *A. oryzae* (SEQ ID No. 1), the *pepE* gene from *A. oryzae* (SEQ ID No. 3)or functional alleles thereof. Also encompassed by the invention are the corresponding PepC and PepE proteases and their production, preferably by recombinant means.

In this aspect the invention relates to processes for the production of the PepC protease or PepE protease from *A. oryzae* comprising transforming a suitable host with a DNA construct comprising a DNA sequence encoding the protease of interest, selecting a transformant capable of producing the protease, cultivating the transformant in an appropriate growth medium and recovering the PepC or PepE protease from the culture.

The host used in such a process is preferably a host according to the above mentioned aspects of the invention

In certain embodiments of the process for producing the PepC or PepE protease the host is *A. oryzae*. In that case it is preferred that the DNA construct comprising a DNA sequence coding for the protease, provides for an extra copy of the gene already present in the host.

The DNA construct comprising the DNA sequence encoding the protease will normally also comprise regulatory elements in order to provide for proper expression and processing of the protease in the host.

The invention is explained in further detail in the Examples given below. These should, however, not in any way be construed as limiting the scope of the invention as defined in the appended claims.

EXAMPLES

Materials and Methods

Strains

A. oryzae, IFO4177: available from Institute for Fermentation, Osaka; 17-25 Juso Hammachi 2-Chome Yodogawa-Ku, Osaka, Japan.

ToC913: The construction of this strain is described in the Examples.

Genes

areA: This gene codes for a regulatory protein controlling nitrogen catabolism.

pepC: This gene codes for a serine protease of the subtilisin type

pepE: This gene codes for an aspartic protease.

pyrG: This gene codes for orotidine-5'-phosphate decarboxylase, an enzyme involved in the biosynthesis of uridine.

bar: This gene was originally isolated from *Streptomyces hygroscopicus* and codes for phosphinothricin acetyltransferase. The enzyme modifies phosphinothricin (=glufosinate) and thereby inactivates this compound which is toxic to bacteria, fungi and plants.

Plasmids

pUC118: Viera and Mesing *J. Meth. Enzymol.* 1987 153 3-11

pSO2: The construction of this plasmid is described in the Examples.

pJers4: A 2.0 kb subclone of pSO2 in pUC118. pJers4 contains a functional *A. oryzae pyrG* gene.

pSO5: The construction of this plasmid from pSO2 is described in the Examples.

pToC56: The construction of this plasmid is described in EP publication No. 0 98 993.

pToC68: The construction of this plasmid is described in WO 91/17243.

pToC90: A subclone of p3SR2, harboring the amdS gene from *Aspergillus nidulans* as a 2.7 kb XbaI fragment [Corrick et al., GENE 1987 **53** 63-71], on a pUC19 vector[Yannisch-Perron et al., GENE 1985 **33** 103-119], prepared as described in WO 91/17243.

pToC266: The construction of this plasmid is described in the Examples.

pToC299: The construction of this plasmid is described in the Examples.

pToC338: The construction of this plasmid is described in the Examples.

pMT1606: The construction of this plasmid from pBP1T (B. Straubinger *et al.* Fungal Genetics Newsletter **39**(1992):82-83) and p775 (EP publication No. 0 98 993) is described in the Examples.

p775: The construction of this plasmid is described in EP publication No. 0 98 993.

p777: The construction of this plasmid is described in EP publication No. 0 98 993.

pHW470: The construction of this plasmid is described in the Examples.

Example 1

Cloning and deletion of the *A. oryzae pepE* gene.

The *A. oryzae pepE* gene was cloned by cross-hybridization with the *A. niger* gene. A partial *A. niger* gene was obtained as a 700 bp PCR fragment from a PCR reaction with *A. niger* chromosomal DNA and *pepE* specific primers made according to the *pepE* sequence published by G. Jarai et al, Gene 145 (1994) 171-178. The fragment was shown to contain *pepE* sequences by DNA sequencing. It hybridizes to *A. oryzae*

chromosomal DNA under stringent conditions and Southern analysis showed that *A. oryzae* contains a single *pepE* like gene.

The *pepE* gene was deleted both by the gene replacement method and the two step gene replacement method (G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992) pp. 1-25. Eds. J. R. Kinghorn and G. Turner; Blackie Academic and Professional). As marker was used the *A. oryzae pyrG* gene, the *A. oryzae* strain was a *pyrG-* strain made by deletion of the *pyrG* gene.

Cloning of the *A. oryzae pepE* gene

A cosmid library of *Aspergillus oryzae* was constructed essentially according to the instruction from the supplier (Stratagene) of the "SuperCos1 cosmid vector kit".

Genomic DNA of *A. oryzae* IFO4177 was prepared from protoplasts made by standard procedures (Christensen, T., et. al., Biotechnology 6 (1988) 1419-1422). After isolation of the protoplasts they were pelleted by centrifugation at 2500 rpm for 5 minutes in a Labofuge T (Heto), the pellet was suspended in 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 µg/ml proteinase K and 0.5% SDS as stated in the manual from the Supercos 1 cosmid vector kit and the rest of the DNA preparation was done according to the kit´s instructions. The size of the genomic DNA was analysed by electrophoresis using the CHEF-gel apparatus from Biorad. A 1% agarose gel was run for 20 hours at 200 volt with a 10-50 second pulse. The gel was stained by etidium bromide and photographed. The DNA was 50->100 kb in size. The DNA was partially restricted by *Sau3A*. The size of the restricted DNA was 20-50 kb determined by the same type of CHEF-gel analysis as above. The CsCl gradient banded SuperCos1 vector was prepared according to the manual. Ligation and packaging was likewise done as described. After titration of the library all of the packaging mix from one ligation and packaging was transfected into the host cells XL1-Blue MR and plated on 50 µg/ml ampicillin LB plates. Approximately 3800 colonies were obtained. Cosmid preparation from 10 colonies showed that they all had inserts of the expected

size. The colonies were picked individually and inoculated in microtiter plate wells with 100 µl LB (100 µg/ml ampicillin) and incubated at 37°C over night. 100 µl of 50% glycerol was added to each well and the whole library was frozen at -80°C. A total of 3822 colonies were stored. This represents the *A. oryzae* genome apr. 4.4 times.

The individually frozen colonies in the library were inoculated onto LB-plates (100µg/ml ampicillin) by using a multipin device with 6 times 8 pins fitting into half a microtiter dish. Plates were made containing colonies from all clones in the library. The plates were incubated at 37°C over night. Sterilized Whatman 540 filters cut to the size of a petri dish were placed upon the colonies which were incubated for two more hours at 37°C. The filters were transferred to LB plates containing 200µg/ml of chloramphenicol and the plates were incubated over night at 37°C.The next day the filters were washed twice in 0.5 M NaOH for 5 minutes, then twice in 0.5 M Tris-HCl (pH=7.4) for 5 minutes and then twice in 2 x SSC for 5 minutes. The filters were wet with ethanol and air-dried.

The filters were hybridized with a 0.7 kb ^{32}P labelled PCR fragment containing part of the *pepE* gene from *A. niger*. The PCR fragment was obtained by running PCR on chromosomal DNA from *A. niger* with two primer 700 bp apart in the DNA sequence. The hybridization was carried out for 16 hours at 65°C in 10 x Denhart, 5 x SSC, 0.02 M EDTA, 1% SDS, 0.15 mg/ml polyA and 0.05 mg/ml yeast tRNA. After hybridization the filters were washed in 2 x SSC, 0.1% SDS at 45°C twice and placed on X-ray films. 5 colonies hybridized with the probe, 4 of them were subsequently shown to contain the *A. oryzae pepE* gene by Southern analysis of the isolated cosmid DNA, using the same probe. Three of the cosmids were identical, thus two different cosmid clones containing *pepE* were isolated, they were called 7C7 and 33C1, names referring to their position in the stored library. Two overlapping fragments, a 4.3 kb *EcoRI* fragment (pToC299) and a 2.4 kb *HindIII* (pToC301) fragment, were subcloned and partially sequenced. SEQ. ID No. 1 shows the DNA sequence and the deduced

aa sequence for the protease. The gene shows strong homology to the *A. niger* gene.

Cloning of the *A. oryzae pyrG* gene

The *A. oryzae pyrG* gene was cloned by cross hybridization with the *A. niger pyrG* gene (W. van Hartingsveldt et al., Mol. Gen. Genet 206:71-75 (1987)). A lambda library of partial *SauIIIA* digested *A. oryzae* IFO4177 DNA was probed at low stringency with a 1 kb DNA fragment from the *A. niger pyrG* gene. A 3.8 kb *HindIII* fragment from a positive clone was subcloned into a pUC118 vector. The resultant plasmid, pSO2, was shown to contain the *pyrG* gene by complementation of an *A. niger pyrG* mutant.

Construction of an *A. oryzae pyrG* minus strain

A *pyrG* deletion plasmid, pSO5, containing about 1 kb of *pyrG* flanking sequences on each end was constructed from the plasmid pSO2. *A. oryzae* IFO4177 was transformed with this construct and transformants were selected by resistance to 5-fluoro-orotic acid (FOA), a phenotype characteristic of *pyrG* mutants. One transformant, HowB101, was shown by Southern analysis to have the expected deletion at the *pyrG* locus. Being a *pyrG* mutant HowB101 requires uridine for growth. HowB101 can be transformed with the *wt pyrG* gene by selection for ability to grow without uridine.

The steps involved in the construction of HowB101 are illustrated in Fig. 1.

Deletion of the *pepE* gene in *A. oryzae*. By the gene replacement method

A plasmid, pToC345, designed to replace the *pepE* gene with the *pyrG* gene, was constructed.

Two PCR reactions were run with pToC299 as template; the first primer set was :

19819 GAAGATCTGCGCGGATGTACATTGTAG

19821 TTAGTCAGAAATTCGTCCCG

The second was :

19820 CCCAAGCTTCATGCTCGACCAGGGCCTCCT

19818 GGTCTGTGTTAACCAAAGAAC

The appr. 800 bp fragment obtained with 19819/19821 was cut with *BglII/HindIII* and cloned together with the 1.1 kb fragment obtained with 19820/19818 and cut with *HindIII/PstI* into *BglII/PstI* cut pIC19R (J. L. Marsh et al, Gene 32 (1984) 481-484). The resulting plasmid was cut at the unique *HindIII* site, dephosphorylated and the 3.5 kb *pyrG* containing fragment from pJaL335 (described in Example 2) was inserted. The construction of pToC345 is illustrated in Figure 2.

HowB101 was transformed with *EcoRI* cut pToC345 using standard procedures and transformants were selected by their ability to grow without the addition of uridine. 100 transformants were reisolated once through conidiospores. Spores were picked from single colonies on the reisolation plates and suspended in 100 ml of water with 0.01% Trition X-100. 1 ml spore suspension from each transformant and from IFO4177, which was included as a control strain, were spotted on two Whatmann 540 filters placed on top of each their YPD plate. The plates were incubated at 30°C for 18 hours. The filters were removed from the plates and placed in 20% SDS for two hours at room temperature. They were then bakes for 3 minutes in a 600W micro-wawe oven. The filters were then washed for 5 minutes in 10%SDS, 2 times 5 minutes in 0.5M NaOH, 1.5M NaCl, one time 5 minutes in 0.5MTris-HCl pH=7.5, 1.5M NaCl and one time 5 minutes in 20xSSC and air dried. The two sets of filters were hybridized by standard procedures with each their ^{32}p-labelled probe. One set was hybridized with a 600bp *BbuI/HindIII* fragment from pToC299 containing the part of the *pepE* gene that was attempted to be deleted. The other set of filters was hybridized with a DNA fragment from the *A. oryzae tpi* gene. Any gene present in one copy, but *pepE* could be used since this is a control of the amount of DNA bound to the filters.

After hybridization the filters were washed with 0.1xSSC, 0.1% SDS at 65°C and the radioactivity bound to the filters were visualized by a PhospoImager. 13 of the transformants were picked for further analysis because they showed little hybridization to the *pepE* probe compared to the hybridization to the control probe. Chromosomal DNA was prepared by standard procedures and a Southern blot of the *EcoRI* restricted DNA was hybridized with a ^{32}P-labelled 1.1kb *BbuI* fragment from pToC299 containing the 3´ part of the *pepE* gene which was not to be deleted. In the *wt* strain a 4.3 kb fragment should hybridize to the probe, in a correct replacement strain the 4.3 kb fragment should be replaced by a 7.2kb fragment. Two of the transformants looked correct, one had no hybridizing bands at all and most had the *wt* band plus maybe one other band, indicating integration of the transforming DNA at a non-homologous locus.

In order to isolate a *pyrG*$^-$ derivative of the *pepE* deleted strain 10^7 conidiospores were spread on FOA containing plates and resistant colonies were selected. The FOA resistant colonies were reisolated, DNA was prepared and Southern analysis was performed to identify the strains in which the *pyrG* gene was lost via recombination between the repeat sequences flanking the gene in pToC345.

Deletion of the *pepE* gene in *A. oryzae* by the two step gene replacement method

A plasmid, pToC315, designed for a two step gene deletion of the *pepE* gene was constructed. A 1.6 kb *EcoRI/HindIII* (the *HindIII* site was blunt ended by treatment with the Klenow fragment of DNA polymerase) from pToC299 containing sequences upstream from the *pepE* gene was cloned together with a 1.4 kb *SalI/BbuI* (the *BbuI* fragment was blunt ended) containing the 3´end of the *pepE* gene into the *EcoRI/SalI* cut vector pUC19. The resulting plasmid was cut at the unique *HindIII* site in the pUC19 linker, dephosphorylated and the 1.8 kb *pyrG* containing fragment

from pJers4 was inserted. The construction of pToC315 is illustrated in Fig. 3.

HowB101 was transformed with pToC315 using standard procedures and transformants were selected by their ability to grow without the addition of uridine. After reisolation chromosomal DNA was prepared from 12 transformants, the DNA was cut with *Asp718* and analysed by Southern analyses with a *BbuI* fragment from pToC301 containing part of the *pepE* gene as a radioactive labelled probe. One transformant had the plasmid integrated in the endogenous *pepE* gene as revealed by the disappearance of the *pepE* specific Asp718 fragment, which had been replaced by two new bands as predicted if pToC315 had integrated as a single copy by homologous recombination at the *pepE* locus. The transformant was named ToC1089. $5x10^7$ conidiae spores of ToC1089 were spread on plates containing 5-fluoro-orotic acid selecting for loss of the *pyrG* gene. This is the second step in a two step gene deletion, the *pyrG* gene can be lost by recombination with either of two pairs of identical sequences, one of which will result in the deletion of the *pepE* gene as well. The procedure is depicted in Fig. 4. The frequency of 5-fluoro-orotic acid resistance was approximately 10^{-5}. The 5-fluoro-orotic acid resistant colonies were reisolated and a strain deleted for the *pepE* gene was identified by Southern analysis.

EXAMPLE 2

Cloning and disruption of the Aspergillus oryzae serine protease pepC

The *A. oryzae pepC* gene was cloned by cross-hybridization with the *A. niger* gene. The *A. niger* gene was obtained as a 1.1 kb PCR fragment from a PCR reaction with *A. niger* chromosomal DNA and *pepC* specific primers made according to the *pepC* sequence published by Frederick G.D et al. Gene 125 (1993) 57-64. The fragment was shown to contain *pepC* sequences by

DNA sequencing. It hybridizes to *A. oryzae* chromosomal DNA under stringent conditions and Southern analysis showed that *A. oryzae* contains a single *pepC* like gene.

The *pepC* gene was deleted by a two step gene replacement method (G. May in "Applied Molecular Genetics of Filamentous Fungi" (1992) pp. 1-25. Eds. J. R. Kinghorn and G. Turner; Blackie Academic and Professional). As marker was used the *A. oryzae pyrG* gene, the *A. oryzae* strain was a *pyrG*⁻ strain made by deletion of the *pyrG* gene.

Cloning of the *A. niger* serine protease *pepC*

From the published cDNA nucleotide sequence encoding *A. niger pepC* (Frederick G.D et al. Gene 125 (1993) 57-64) two oligonucleotides were designed so that the encoding part of the pepC gene where amplified in a PCR reaction. The primer #5258 (5'-CTAGGATCCAAGGCATTTATGAAGGGCATCCTCGGCCTTTCC) where made so that the 3' end of the nucleotide sequence corresponds to the N-terminal part of the *pepC* gene (underline) and the 5'-end is for facilitating cloning (contains a *BamHI* restriction endonuclease site). The primer #5259

(5'-CTACTCGAGTCAAAAAAAAACCAAGTCTTCCGATCTACG)

where made so that the 3' end of the nucleotide sequence corresponds to the C-terminal part of the *pepC* gene and the 5'-end is for facilitating cloning (contains a *XhoI* restriction endonuclease site).

Genomic DNA from *A. niger* was used as template in the PCR reaction. Amplification reaction were performed in 100 µl volumes containing 2.5 units Taq-polymerase, 100 ng of *A. niger* genomic DNA, 50 mM KCl, 10 mM Tris-HCl pH 8.0,1.5 mM $MgCl_2$, 250 nM of each dNTP, and 100 pM of each of the two primers described above.

Amplification was carried out in a Perkin-Elmer Cetus DNA Termal 480, and consisted of one cycle of 3 minutes at 94°C,

followed by 25 cycles of 1 minutes at 94°C, 30 seconds at 55°C, and 1 minutes at 72°C. The PCR reaction produces one DNA fragment of ca. 1.1 kb in length. This fragment were isolated by gel electrophoresis, purified, cloned into the vector pCR®II (Invitrogen Corporation), and sequenced using standard methods known in the art of molecular biology. The resulting plasmid were called pJaL197.

Cloning of the *A. oryzae* serine protease pepC

Southern blot of genomic DNA from *A. oryzae* IFO4177 where hybridized with the 1.1 kb ^{32}P labelled *EcoRI* DNA fragment from pJaL197 clone containing the *A. niger pepC* gene. Genomic DNA was cut with the following restriction enzymes: *EcoRI*, *BamHI*, *XhoI*, and *HindIII*. Hybridization was carried out for 16 hours at 65°C in 10 * Denhart, 5 * SSC, 0.02 M EDTA, 1% SDS, 0.15 mg/ml polyA and 0.05 mg/ml yeast tRNA. After hybridization the filters were washed in 2 x SSC, 0.1% SDS at 65°C twice and placed on X-ray films. The probe hybridized to a single size of fragment in each of the four digest, indicating that the *pepC* gene is present in a single copy in *A. oryzae* IFO4177.

A partially library of *A. oryzae* genomic DNA was constructed containing *BamHI* fragments with a size of 4.5-5.5 kb and ligated into the vector pIC19H. The above *A. niger pepC* gene clone was radiolabelled and used to probe the partial *A. oryzae* *BamHI* genomic library. Hybridization was carried out as described above. About 4000 *E. coli* colonies were screened and four positive colonies was obtained. The 4 clones was shown to be identical by restriction enzyme digestion One of these clones called pJaL235 (Fig. 5), with an insert of 4.6 kb, was analyses further by restriction mapping and Southern blotting. This shows that the *pepC* gene is located in a 2.9 kb *BamHI/SalI* fragment. Sequencing of this 2.9 kb *BamHI/SalI* fragment revealed the presence of a long open reading frame of 495 amino acids interrupted by two introns with consensus sequences indicative of

intron splicing. The sequence of the *A. oryzae pepC* gene is shown in SEQ ID No. 3.

Construction of *A. oryzae pyrG* gene flanked by a repeated sequence

By PCR, with the primer #7659

(5'-GGAGGAAGATCTCTCTGGTACTCTTCGATCTC),

where the 3' end of the nucleotide sequence corresponds to position 7-26 in pSO2 (underline) and the 5'-end is for facilitating cloning (contains a *BglII* restriction endonuclease site), and the primer #7656

(5'-GGAGGAGAATTCAAGCTTCTTCTACATCACAGTTTGAAAGC),

where the 3' end of the nucleotide sequence corresponds to position 385-407 in pSO2 (underline) and the 5'-end is for facilitating cloning (contains a *EcoRI* and *HindIII* restriction endonuclease site), on the plasmid pSO2 a 432 bp fragment was amplified. The fragment was digested with *BglII* and *EcoRI* and isolated by gel electrophoresis, purified, and cloned into the corresponding site in pSO2, resulting in plasmid pJaL335 (The construction is outlined in Fig. 6.

Construction of *A. oryzae pepC* disruption plasmid

Plasmid pJaL235 was digested with *PvuI* and treated with Klenow polymerase to make the ends blunt and then digested with *HindIII*. The 2.6 kb fragment were isolated by gel electrophoresis, and purified. The 2.6 kb fragment was cloned into pUC12 digested with *SmaI* and *HindIII* giving plasmid pJaL308.

Plasmid pJaL308 was digested with *SmaI* and treated with bacterial alkaline phosphatase to remove the 5' phosphate groups according to the manufacturers instructions and phenol extracted and precipitated.

Plasmid pS02 was digested with *HindIII*, and treated with Klenow polymerase to make the ends blunt. The 3.8 kb fragment encoding the *A. oryzae pyrG* gene were isolated by gel electrophoresis, and purified.

The two fragments are mixed together and ligated. After transformations of *E. coli*, the colonies carrying the correct plasmids are identified by restriction enzyme digestion of mini-plasmid preparations. The construction of pJaL363 is illustrated in Fig. 7.

Plasmid pJaL363 consist of pUC12 vector containing a fragment which carries the *pepC* gene flanked by an *EcoRI* site and an *HindIII* and where the *pepC* is interrupted by an 3.8 kb DNA fragment encoding the *A. oryzae pyrG* gene.

Plasmid pJaL335 is digested with *HindIII*, and treated with Klenow polymerase to make the ends blunt. The 3.5 kb fragment encoding the *A. oryzae pyrG* gene is isolated by gel electrophoresis, and purified. The fragment is cloned into pJaL308 *SmaI* restriction site. The construction of pJaLz is outlined in Fig. 8. The plasmid consist of pUC12 vector containing a fragment which carries the *pepC* gene flanked by an *EcoRI* site and an *HindIII* and where the *pepC* is interrupted by an 3.5 kb DNA fragment encoding the *A. oryzae pyrG* gene.

Transformation of *A. oryzae* strain HowB101

15 µg of either one of the disruption plasmids is digested to completion by *HindIII* and *EcoRI*. The completeness of the digest is checked by running an aliquot on a gel and the remainder of the DNA is phenol extracted, precipitated and resuspended in 10 µl of sterile water.

The transformation of *A. oryzae* HowB101 host strain is preformed by the protoplast method (Christensen et al. Biotechnology (1988) 6:1419-1422). Typically, *A. oryzae* mycelia is grown in a rich nutrient broth. The mycelia is separated from the broth by filtration. The enzyme preparation Novozyme® (Novo Nordisk) is added to the mycelia in osmotically stabilizing buffer such as

1.2 M $MgSO_4$ buffered to pH 5.0 with sodium phosphate. The suspension was incubated for 60 minutes at 37°C with agitation. The protoplast is filtered through mira-cloth to remove mycelial debris. The protoplast is harvested and washed twice with STC (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). The protoplast is finally resuspended in 200-1000 µl STC.

For transformation 5 µg DNA is added to 100 µl protoplast suspension and then 200 µl PEG solution (60% PEG 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) is added and the mixture is incubated for 20 minutes at room temperature. The protoplast is harvested and washed twice with 1.2 M sorbitol. The protoplast is finally resuspended 200 ml 1.2 M sorbitol, plated on selective plates (minimal medium + 10 g/l Bacto-Agar (Difco), and incubated at 37°C. After 3-4 days of growth at 37°C, stable transformants appear as vigorously growing and sporulating colonies.

Identification of a *pepC* deletion strain

From the stable colonies individual spores is streaked on fresh minimal plates. Single colonies is selected and restreaked to give pure cultures. These are used to inoculate 10 ml of liquid YPM medium (1% yeast extract, 1% peptone, 2% maltose). After 18 hours at 30°C shaking at 180 rpm, the mycelia are harvested on filter paper. Mycelia is then transfer to an 2 ml eppendorf tube and freeze dried. After freeze drying DNA is prepared from the individual mycelia by grinding the mycelia to a fine powder with a pestle in the tube. This powder are resuspended in 0.5 ml of 50 mM EDTA pH 8.0, 0.2% SDS, 1 µl DEP by vortexing. These are incubate at 65°C for 20 minutes. After this is added 0.1 ml 5 M KAc pH 6.5, the solution is mixed and incubated on ice for 5 minutes. The cell debris is separated from the DNA solution by centrifugation at 20.000 rpm for 5 minutes. 0.4 ml supernatant are precipitated with 0.3 ml isopropanol and centrifugated at 20.000 rpm for 10 minutes. The DNA pellet is redissolved in 100 µl of sterile TE buffer containing 0.1 mg/ml RNAaseA.

3 µg of each DNA is digested with *EcoRI*, fractionated by agarose gel electrophoresis, transferred to Immobilan-N membrane

filters, and probe with the 1.5 kb ^{32}P labelled *NcoI* DNA fragment from pJaL335 containing part of the *pepC* protease gene. Strains which carry a disruption of the *pepC* are easily recognized by that the wild type band on 3.6 kb is shifted to a 7.4 kb band in the transformant.

The disrupted *A. oryzae pepC* strain is made *pyrG* minus by selecting spontaneous mutant resistance to 5-fluoro-orotic acid, a phenotype characteristic of *pyrG* mutants. Being a *pyrG* mutant the strain requires uridine for growth. The strain can be transformed with the *wt pyrG* gene by selection for the ability to grow without uridine.

EXAMPLE 3

Construction of an *Aspergillus oryzae areAΔ* strain

The *areAΔ* strain was constructed as follows. The *areA* gene from *A. oryzae* was cloned. A *pyrG*⁻ strain also deficient for either *pepC* or *pepE* or *pepC* plus *pepE* was transformed with a plasmid carrying the *pyrG* gene inserted between DNA fragments upstream and downstream from the *areA* gene. The coding region for *areA* was not present on the plasmid. Transformants were selected for their ability to grow in the absence of uridine and in the presence of chlorate. This double selection selects both for a functional *pyrG* gene and for *areA* minus. Strains obtained by this selection procedure were finally screened by Southern analysis to identify those in which the chromosomal *areA* gene was substituted by the *pyrG* gene.

Cloning of the *areA* gene

The *A. oryzae areA* gene was cloned by cross hybridization to the *A. nidulans areA* gene (B. Kudla et al., EMBO J. 9:1355-1364 (1990)). A genomic library of *A. oryzae* IFO4177 was prepared by partial digestion of chromosomal DNA with *SauIIIA* and cloning of the obtained DNA fragments into the vector lGEM-II

(obtained from Promega). Cross hybridization of the library with the *A. nidulans areA* gene was performed in 40% formamide at 37°C. Hybridizing 1 clones were isolated and from these fragments were sub-cloned into the vector pBluescript SK+ (obtained from Stratagene) giving rise to the plasmids pSK5 and pSK9 illustrated in Fig. 9. The cloned gene was able to complement an *A. nidulans areA* mutant, proving that it is indeed the *A. oryzae areA* homolog. 5643 bp of the clone was sequenced, and comparison of the sequences of the *A. oryzae* and the *A. nidulans areA* genes shows that they are highly homologous. The sequence of the *A. oryzae areA* gene is shown in SEQ ID No. 5.

Construction of the *areA* deletion plasmid

In order to delete the *areA* gene from the *A. oryzae* chromosome the plasmid pToC266 was constructed. pToC266 contains a 2.1 kb DNA fragment originating upstream of the *areA* gene (isolated from pSK5) and a 1.4 kb DNA fragment originating downstream from the *areA* gene (isolated from pSK9). The two fragments are separated by approximately 3.2 kb in the genome, the coding region is situated in this part of the gene. The *A. oryzae pyrG* gene from pJers4 was inserted between the *areA* upstream and downstream DNA fragments. The construction of pToC266 is illustrated in Figs. 10a and 10b. pToC266 has a unique *EcoRI* site and was linearized by cutting with this restriction enzyme before used in transformations.

Selection of *A. oryzae areA* strains

A *pyrG* strain also deficient for either *pepC* or *pepE* or *pepC* plus *pepE* is transformed with linearized pToC266. Transformants are selected on minimal plates (Cove *Biochem. biophy. Acta* (1966) 113 : 51-56) containing glutamine as the nitrogen source and glucose as the carbon source. Transformants are reisolated twice on the same type of plates, and then subjected to growth test on different nitrogen sources. Transformants growing well on glutamine but not on nitrate, ammonium or urea are expected to be deleted for *areA*. The deletion is confirmed by Southern analysis.

EXAMPLE 4

Construction of pMT1606

A plasmid containing the *bar* gene from *Streptomyces hygroscopius* (C. J. Thompson et. al, EMBO J. 6 : 2519-2523 (1987)) inserted after the *A. oryzae* TAKA-amylase promoter and followed by a fragment containing the transcriptional terminator and polyadenylation signal from the *A. niger gla* gene was constructed.

The plasmid, pMT1606, can be used for selection of glufosinate resistant transformants of *A. oryzae*. pMT1606 was constructed by isolating the *bar* gene from the plasmid pBP1T (B. Straubinger et. al, Fungal Genetics Newsletter 39 : 82-83 (1992)) and cloning it into the fungal expression plasmid p775 described in EP publication No. 0 098 993 A1. Fig. 11 illustrates the construction of pMT1606.

EXAMPLE 5

Production of chymosin in *A. oryzae* (*areAΔ,pepEΔ,pepC*$^-$)

An *A. oryzae areAΔ,pepEΔ,pepC*$^-$ strain is transformed with the plasmid pToC56 (Fig. 12), which is a fungal expression plasmid for the mammalian enzyme chymosin, by co-transformation

with pMT1606. Construction of the plasmid pToC56 is described in EP publication No. 0 98 993.

Transformants are selected for growth on minimal medium containing 10 mM ammonium and 1 mg/ml glufosinate and screened for the presence of pToC56 by the ability to produce chymosin. The transformants are grown in shake flasks in minimal medium containing maltodextrin and glutamine for 4 days at 30°C.The content of chymosin in the supernatants were analysed by SDS-Page and Western blotting.

Example 6

Production of PepC in *A.oryzae*

Construction of an expression plasmid for *pepC*.

Plasmid pJaL235 was digested with *AatII* and *NsiI* and treated with Klenow polymerase to make the ends blunt. The 1.7 kb fragment was isolated by gel electrophoresis, and purified. The 1.7 kb fragment was cloned into pIC19H digested with *SmaI* giving pJaL365.

Plasmid pJaL365 was digested with *BamHI* and *XhoI* and the 1.7 kb fragment was isolated by gel electrophoresis, and purified. The 1.7 kb fragment was cloned into pToC68 digested with *BamHI* and *XhoI* giving pJaL368 (Figs. 13a and 13 b).

An *A. oryzae* strain is transformed with the plasmid pJaL368, which is a fungal expression plasmid for the protease PepC, by cotransformation with pToC90.

Transformants are selected for growth on minimal medium containing 10 mM acetamide and screened for the presence of pJaL368 by the ability to produce the protease PepC.

Example 7

Overexpression of *pepE*

A plasmid called pToC338 carrying the *pepE* gene fused to the TAKA-amylase promoter from *A. oryzae* was constructed. Figs. 14a and 14b depicts the construction.

An *EcoRI/SalI* fragment from pToC299 containing most of the coding region and appr. 430 bp of the 3´ untranslated region of *pepE* was cloned into *EcoRI/BamHI* cut pUC19 together with a synthetic DNA fragment of the following sequence :

8681 GATCCACCATGAAG
8747 GTGGTACTTCAGCT

The resulting plasmid called pToC334 was cut with *BamHI/EcoRI* and a fragment containing the entire structural gene of *pepE* with a *BamHI* site fused immediately upstream of the start codon was isolated, approximately 430 bp of untranslated 3´ sequence was also present in the fragment. The fragment was cloned into *EcoRI/SalI* cut pUC19 together with an approximately 1.1 kb *SalI/BamHI* fragment from the plasmid p775 containing the TAKA-amylase promoter from *A. oryzae*. The resulting plasmid was named pToC338.

pToC338 was co-transformed into *A. oryzae* JaL125 (an *A. oryzae alp* minus strain described in Danish Patent Application No. 0354/96) with pToC90, which contains the *A. nidulans* acetamidase (*amdS*) gene, using standard procedures (*e.g.* as described in EP 0 098 993 A1). Transformants were selected by their ability to use acetamide as the sole nitrogen source. 11 transformants were reisolated twice through conidiospores. The transformants were fermented for three days at 30°C in10 ml YPM (YP with 2% maltose) and the fermentation broth was analysed by SDS-page. One of the transformants produced a protein of the same size as the protein encoded by the *pepE* gene, protease activity measurements confirmed that the broth from that transformant contained a higher activity toward casein at pH=5.5 compared to the host strain JaL125. The protein was purified and N-terminal sequencing showed that it is indeed the protein encoded by the *pepE* gene. The N-terminal of the secreted protein was:

gly*-arg-his-asp-val-leu-val-asp-asn-phe-leu-asn-ala-gln-tyr-phe-ser-glu-ile-glu-ile-gly-thr-pro-pro-gln-lys-phe-lys

*this residue could also be a lysine.

confirming the expression of the PepE protease.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
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(ii) TITLE OF INVENTION: Novel Microorganisms

(iii) NUMBER OF SEQUENCES: 12

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2454 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus oryzae
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:603..701
(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:702..791
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:792..942

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:943..1001
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1002..1656
(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:1657..1713
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1714..2001
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(603-701, 792-942, 1002-1656, 1714-2001)
(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:603..2001

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATCAGCTCCA CACCCTTGGA TAGTGATAAG AGACACAAGG AGTTCTGAGT ATGGTATTAT     180

AAGTGCAGTG AGTTGGGATG AAACAGAGAG ATAGAGGGAA TACTCCTATT TATCAATGAA     240

CGTATACAGA CATACCCCAG CAGCGTTCCT GGCGGTATTG TAAAAGGGCC GTACCTTGGA     300

GATCAAGTGA TGAGACACCC GTGATGCAGG AACTCCACTT CAATCCAATG ACGCATCGAG     360

TTGCTCCCTG ATTGGTTGAT ACGCAGGTCG CTCCGCAACC GGTCCGCATC ACCTCACTTC     420

CCTCCCCCAG ACCTGGAGGT ACCTCTCCCG TCCTTCTCTC CCTCTCCATC CCATCATCTA     480

TCCCTCTCCA GACCCTGATT GTATTTCATC ATTCCTATCG TCCCATATTA ATAGAGTATT     540

GCTAGTTTTC TTTTGATTTC GTCTGTTGAG GTGCTGCTTT TTTGTCGCCG TTGTCGCCCA     600

CC ATG AAG TCG ACC TTG GTT ACG GCC TCT GTG CTG TTG GGC TGT GCT         647
   Met Lys Ser Thr Leu Val Thr Ala Ser Val Leu Leu Gly Cys Ala
     1               5                  10                  15

TCC GCC GAG GTT CAC AAG CTG AAG CTC AAC AAG GTG CCC GTG TCC GAG       695
Ser Ala Glu Val His Lys Leu Lys Leu Asn Lys Val Pro Val Ser Glu
                 20                  25                  30

CAA TTT GTGAGTAGAC CTTACTATTC CGGCCATGAA AATATTCATC TACCCATCTG        751
Gln Phe

AAAGCTTGTC GGGACGAATT TCTGACTAAA TCGTATCCAG AAC TTG CAC AAC ATC       806
                                        Asn Leu His Asn Ile
                                             35
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GAC ACC CAT GTG CAG GCT CTC GGC CAG AAG TAC ATG GGA ATC CGT CCC        854
Asp Thr His Val Gln Ala Leu Gly Gln Lys Tyr Met Gly Ile Arg Pro
     40                  45                  50

AAC ATC AAG CAA GAT CTT CTC AAT GAG AAC CCG ATT AAC GAT ATG GGA        902
Asn Ile Lys Gln Asp Leu Leu Asn Glu Asn Pro Ile Asn Asp Met Gly
 55                  60                  65                  70

CGT CAT GAT GTC CTT GTT GAC AAC TTC CTG AAT GCA CAA T                  942
Arg His Asp Val Leu Val Asp Asn Phe Leu Asn Ala Gln
                 75                  80

GTACGAAACC CTAGTAATAC TTGAAGGGGG GCTCCAACTT ACGCGTAGAT TCTCTAAAG  AC 1003
                                                                  Tyr

TTC TCC GAA ATC GAG ATC GGT ACT CCT CCA CAG AAG TTC AAG GTG GTC        1051
Phe Ser Glu Ile Glu Ile Gly Thr Pro Pro Gln Lys Phe Lys Val Val
 85                  90                  95                  100

CTT GAC ACT GGC AGC TCA AAC CTA TGG GTG CCC TCT TCG GAG TGT GGT        1099
Leu Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser Ser Glu Cys Gly
                105                 110                 115

TCT ATC GCC TGC TAT TTG CAT AAC AAG TAC GAC TCA TCC TCG TCC TCC        1147
Ser Ile Ala Cys Tyr Leu His Asn Lys Tyr Asp Ser Ser Ser Ser Ser
            120                 125                 130

ACG TAC CAG AAG AAT GGC AGC GAA TTT GCC ATC AAG TAC GGC TCT GGT        1195
Thr Tyr Gln Lys Asn Gly Ser Glu Phe Ala Ile Lys Tyr Gly Ser Gly
        135                 140                 145

AGC CTG AGT GGT TTT GTT TCT CAG GAT ACT CTC AAG ATC GGT GAC CTG        1243
Ser Leu Ser Gly Phe Val Ser Gln Asp Thr Leu Lys Ile Gly Asp Leu
    150                 155                 160

AAG GTG AAG GAT CAG CTG TTC GCC GAG GCT ACT AGT GAG CCC GGC CTT        1291
Lys Val Lys Asp Gln Leu Phe Ala Glu Ala Thr Ser Glu Pro Gly Leu
165                 170                 175                 180

GCT TTT GCC TTT GGC CGC TTT GAT GGT ATC CTT GGG TTG GGA TTT GAC        1339
Ala Phe Ala Phe Gly Arg Phe Asp Gly Ile Leu Gly Leu Gly Phe Asp
                185                 190                 195

ACA ATT TCC GTC AAC AAG ATT CCT CCA CCC TTC TAT AGC ATG CTC GAC        1387
Thr Ile Ser Val Asn Lys Ile Pro Pro Pro Phe Tyr Ser Met Leu Asp
            200                 205                 210

CAG GGC CTC CTC GAC GAG CCA GTC TTT GCT TTC TAC CTT GGA GAC ACT        1435
Gln Gly Leu Leu Asp Glu Pro Val Phe Ala Phe Tyr Leu Gly Asp Thr
        215                 220                 225

AAC AAG GAA GGT GAT GAC TCC GTA GCG ACA TTC GGC GGT GTT GAC AAG        1483
Asn Lys Glu Gly Asp Asp Ser Val Ala Thr Phe Gly Gly Val Asp Lys
    230                 235                 240

GAT CAC TAC ACC GGC GAG TTG GTC AAG ATT CCC CTT CGC CGC AAG GCC        1531
Asp His Tyr Thr Gly Glu Leu Val Lys Ile Pro Leu Arg Arg Lys Ala
245                 250                 255                 260

TAC TGG GAG GTT GAC CTT GAT GCT ATC GCC CTT GGC GAT AGC GTT GCT        1579
Tyr Trp Glu Val Asp Leu Asp Ala Ile Ala Leu Gly Asp Ser Val Ala
                265                 270                 275

GAA CTC GAT AAC ACC GGT GTC ATT CTG GAT ACC GGC ACT TCC CTT ATC        1627
Glu Leu Asp Asn Thr Gly Val Ile Leu Asp Thr Gly Thr Ser Leu Ile
            280                 285                 290
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GCC TTG GCC ACC ACC CTT GCC GAG CTT AT  GTAAGTCAAG CCAGTGTACT          1676
Ala Leu Ala Thr Thr Leu Ala Glu Leu Ile
        295                 300

GTGCATGTCT GTCATACTCT TACTAACTAT TCTGAAG T AAC AAG GAA ATC GGT         1729
                                        Asn Lys Glu Ile Gly
                                            305

GCC AAG AAG GGC TTC ACC GGC CAA TAC TCG GTT GAC TGT GAC AAG CGC        1777
Ala Lys Lys Gly Phe Thr Gly Gln Tyr Ser Val Asp Cys Asp Lys Arg
        310                 315                 320

GAT TCC TTG CCT GAC CTC ACC TTC ACC CTG AGC GGA TAC AAC TTC ACC        1825
Asp Ser Leu Pro Asp Leu Thr Phe Thr Leu Ser Gly Tyr Asn Phe Thr
    325                 330                 335

ATT GGT CCC TAC GAC TAC ACT CTT GAA GTC CAG GGA TCT TGC ATC AGC        1873
Ile Gly Pro Tyr Asp Tyr Thr Leu Glu Val Gln Gly Ser Cys Ile Ser
340                 345                 350                 355

GCC TTC ATG GGC ATG GAC TTC CCT GAA CCC GTT GGC CCC TTG GCC ATC        1921
Ala Phe Met Gly Met Asp Phe Pro Glu Pro Val Gly Pro Leu Ala Ile
                360                 365                 370

CTG GGT GAC GCG TTC CTC AGG AAG TGG TAC AGT GTG TAC GAC CTC GCC        1969
Leu Gly Asp Ala Phe Leu Arg Lys Trp Tyr Ser Val Tyr Asp Leu Ala
            375                 380                 385

AAC GGT GCT GTT GGC CTG GCC AAG GCT AAG TAACCAAGTA ATCTACCATG          2019
Asn Gly Ala Val Gly Leu Ala Lys Ala Lys
        390                 395

CTATGTTCTT ATTGGTTGCT TGTGTATGTG AGACAATGGT ACATGATAGC CTGCCTCGGT      2079

AGTTGGTTGG CCTTTTTCTG TTACGGGAAA TCGGCAAAGC CTTGTTTTCG CTTATGACCT      2139

CTATCCTGTT TGTTATTGAT ATTTTGTGTG ACTCAGTGAG CCACTGGCTA TGCTCTAATG      2199

ACATTCATTG GATGCCGATA GTTCTATATA CATTGCGATT TTAACGCGTA TCTTTGATCT      2259

ATCGGTACAA TGATTCCCTA CTAAAGGTAG CCCAACTAGA CAACTATGCC TACGACCTCT      2319

CTACATTCTT CATAGCTCCG TGTGGAGTCC GTCTCATACA ACCTCGAGCA ACCTGCAGTT      2379

CTTTGGTTAA CACAGACCAC ACCTTAAAAC GGCACGATCC ATTCGAATAG ACAAGCCCTC      2439

TTAATATTTG AATTC                                                       2454
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 397 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Lys Ser Thr Leu Val Thr Ala Ser Val Leu Leu Gly Cys Ala Ser
 1               5                  10                  15
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Ala Glu Val His Lys Leu Lys Leu Asn Lys Val Pro Val Ser Glu Gln
20 25 30

Phe Asn Leu His Asn Ile Asp Thr His Val Gln Ala Leu Gly Gln Lys
35 40 45

Tyr Met Gly Ile Arg Pro Asn Ile Lys Gln Asp Leu Leu Asn Glu Asn
50 55 60

Pro Ile Asn Asp Met Gly Arg His Asp Val Leu Val Asp Asn Phe Leu
65 70 75 80

Asn Ala Gln Tyr Phe Ser Glu Ile Glu Ile Gly Thr Pro Pro Gln Lys
85 90 95

Phe Lys Val Val Leu Asp Thr Gly Ser Ser Asn Leu Trp Val Pro Ser
100 105 110

Ser Glu Cys Gly Ser Ile Ala Cys Tyr Leu His Asn Lys Tyr Asp Ser
115 120 125

Ser Ser Ser Ser Thr Tyr Gln Lys Asn Gly Ser Glu Phe Ala Ile Lys
130 135 140

Tyr Gly Ser Gly Ser Leu Ser Gly Phe Val Ser Gln Asp Thr Leu Lys
145 150 155 160

Ile Gly Asp Leu Lys Val Lys Asp Gln Leu Phe Ala Glu Ala Thr Ser
165 170 175

Glu Pro Gly Leu Ala Phe Ala Phe Gly Arg Phe Asp Gly Ile Leu Gly
180 185 190

Leu Gly Phe Asp Thr Ile Ser Val Asn Lys Ile Pro Pro Pro Phe Tyr
195 200 205

Ser Met Leu Asp Gln Gly Leu Leu Asp Glu Pro Val Phe Ala Phe Tyr
210 215 220

Leu Gly Asp Thr Asn Lys Glu Gly Asp Asp Ser Val Ala Thr Phe Gly
225 230 235 240

Gly Val Asp Lys Asp His Tyr Thr Gly Glu Leu Val Lys Ile Pro Leu
245 250 255

Arg Arg Lys Ala Tyr Trp Glu Val Asp Leu Asp Ala Ile Ala Leu Gly
260 265 270

Asp Ser Val Ala Glu Leu Asp Asn Thr Gly Val Ile Leu Asp Thr Gly
275 280 285

Thr Ser Leu Ile Ala Leu Ala Thr Thr Leu Ala Glu Leu Ile Asn Lys
290 295 300

Glu Ile Gly Ala Lys Lys Gly Phe Thr Gly Gln Tyr Ser Val Asp Cys
305 310 315 320

Asp Lys Arg Asp Ser Leu Pro Asp Leu Thr Phe Thr Leu Ser Gly Tyr
325 330 335

Asn Phe Thr Ile Gly Pro Tyr Asp Tyr Thr Leu Glu Val Gln Gly Ser
340 345 350

Cys Ile Ser Ala Phe Met Gly Met Asp Phe Pro Glu Pro Val Gly Pro
355 360 365

Leu Ala Ile Leu Gly Asp Ala Phe Leu Arg Lys Trp Tyr Ser Val Tyr
370 375 380

Asp Leu Ala Asn Gly Ala Val Gly Leu Ala Lys Ala Lys
385 390 395

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3224 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iv) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus oryzae
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:388..756
(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:757..817
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:818..1753
(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION:1754..1814
(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION:1815..1997
(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:388..1997
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(388..756, 818..1753, 1815..1997)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGATCCATTA CCCTCTTACC GCCATACCCC AGGTCTTGCG ACCGCGCTAA TCGGGAGCGA 60

TCGACGCGCG GCACCTCCTC AGTAAAGCTG TGTCATCATT GTAAATTACC GTATCCCGGT 120

TGCATCATCC TCCGCTGCCC TTGCCTGCTT GGGGGATCGA CCTATTAAGC CCAGCTATCT 180

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TACACCTGCT CCCATCCTCC TCTTCTCCAA CTCCTCATCC ATCCTTCCTC CTCCTTCTTC      240

CTTTTAACCC CCCCAACTCA GCATCGTTCC ATCCTCCCAT CTTTCCTTTC TTTTTACCTC      300

AAATCTCCAT CTGTATTCTT TCCTCTTAGA ACTCTTCCTT TCCCCCCTTC TGTACCTTGT      360

GTTTAGACGT CACTCTTGTT GCCCATC ATG AGA GGC ATC CTC GGC CTT TCC           411
                              Met Arg Gly Ile Leu Gly Leu Ser
                                1               5

CTG CTG CCA CTA CTA GCA GCG GCC TCC CCC GTT GCT GTT GAC TCC ATC         459
Leu Leu Pro Leu Leu Ala Ala Ala Ser Pro Val Ala Val Asp Ser Ile
     10                  15                  20

CAC AAC GGA GCG GCT CCC ATT CTT TCG GCC TCA AAT GCC AAA GAG GTT         507
His Asn Gly Ala Ala Pro Ile Leu Ser Ala Ser Asn Ala Lys Glu Val
 25                  30                  35                  40

CCA GAC TCT TAC ATT GTC GTC TTC AAG AAG CAT GTT TCC GCT GAA ACG         555
Pro Asp Ser Tyr Ile Val Val Phe Lys Lys His Val Ser Ala Glu Thr
                 45                  50                  55

GCT GCT GCT CAT CAC ACC TGG GTG CAG GAC ATC CAC GAT TCG ATG ACT         603
Ala Ala Ala His His Thr Trp Val Gln Asp Ile His Asp Ser Met Thr
            60                  65                  70

GGA CGC ATC GAC CTG AAG AAG CGC TCT CTT TTT GGT TTC AGT GAT GAC         651
Gly Arg Ile Asp Leu Lys Lys Arg Ser Leu Phe Gly Phe Ser Asp Asp
        75                  80                  85

CTT TAC CTC GGT CTC AAG AAC ACC TTC GAT ATC GCC GGG TCC CTA GCG         699
Leu Tyr Leu Gly Leu Lys Asn Thr Phe Asp Ile Ala Gly Ser Leu Ala
     90                  95                  100

GGC TAC TCC GGA CAT TTC CAT GAG GAT GTG ATC GAG CAG GTC CGG AGA         747
Gly Tyr Ser Gly His Phe His Glu Asp Val Ile Glu Gln Val Arg Arg
105                 110                 115                 120

CAT CCT GAT GTAGGTTCCC CCCTCGGCCC ACCCGTTTTT GTAGAGCCCT                 796
His Pro Asp

TGGTCTAACT TGATTTTCAA G GTT GAA TAC ATC GAG AAA GAC ACC GAA GTC         847
                       Val Glu Tyr Ile Glu Lys Asp Thr Glu Val
                       125                 130

CAC ACC ATG GAG GAG ACA ACC GAG AAG AAT GCT CCC TGG GGC TTG GCT         895
His Thr Met Glu Glu Thr Thr Glu Lys Asn Ala Pro Trp Gly Leu Ala
    135                 140                 145

CGT ATC TCT CAC CGT GAC AGC CTC TCG TTC GGT ACC TTT AAC AAG TAC         943
Arg Ile Ser His Arg Asp Ser Leu Ser Phe Gly Thr Phe Asn Lys Tyr
150                 155                 160                 165

CTG TAT GCT TCG GAA GGC GGT GAG GGT GTC GAT GCT TAT ACT ATT GAC         991
Leu Tyr Ala Ser Glu Gly Gly Glu Gly Val Asp Ala Tyr Thr Ile Asp
                170                 175                 180

ACT GGT ATC AAC ATT GAG CAT GTC GAT TTC GAG GAT CGA GCA CAC TGG        1039
Thr Gly Ile Asn Ile Glu His Val Asp Phe Glu Asp Arg Ala His Trp
            185                 190                 195

GGA AAG ACC ATC CCT AGC AAT GAT GAG GAT GCG GAT GGC AAC GGA CAC        1087
Gly Lys Thr Ile Pro Ser Asn Asp Glu Asp Ala Asp Gly Asn Gly His
        200                 205                 210
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GGA ACT CAC TGC TCC GGA ACC ATT GCT GGT AAG AAG TAC GGT GTT GCC      1135
Gly Thr His Cys Ser Gly Thr Ile Ala Gly Lys Lys Tyr Gly Val Ala
    215                 220                 225

AAG AAG GCC AAC ATC TAT GCC GTC AAG GTC TTG AGG TCC AGC GGT TCT      1183
Lys Lys Ala Asn Ile Tyr Ala Val Lys Val Leu Arg Ser Ser Gly Ser
230                 235                 240                 245

GGC ACT ATG TCC GAT GTC GTT CTG GGT GTC GAG TGG GCC GTC CAG TCC      1231
Gly Thr Met Ser Asp Val Val Leu Gly Val Glu Trp Ala Val Gln Ser
                250                 255                 260

CAC CTC AAG AAG GCT AAG GAC GCC AAA GAT GCC AAG GTC AAG GGT TTC      1279
His Leu Lys Lys Ala Lys Asp Ala Lys Asp Ala Lys Val Lys Gly Phe
            265                 270                 275

AAG GGC AGC GTT GCC AAC ATG AGT CTT GGT GGT GCC AAG TCC AGG ACC      1327
Lys Gly Ser Val Ala Asn Met Ser Leu Gly Gly Ala Lys Ser Arg Thr
        280                 285                 290

CTT GAG GCT GCT GTC AAT GCT GGT GTT GAG GCT GGT CTT CAC TTC GCC      1375
Leu Glu Ala Ala Val Asn Ala Gly Val Glu Ala Gly Leu His Phe Ala
    295                 300                 305

GTT GCT GCT GGT AAC GAC AAT GCC GAT GCC TGC AAC TAC TCC CCT GCT      1423
Val Ala Ala Gly Asn Asp Asn Ala Asp Ala Cys Asn Tyr Ser Pro Ala
310                 315                 320                 325

GCC GCT GAG AAT GCC ATC ACT GTC GGT GCC TCG ACC CTT CAG GAT GAG      1471
Ala Ala Glu Asn Ala Ile Thr Val Gly Ala Ser Thr Leu Gln Asp Glu
                330                 335                 340

CGT GCT TAC TTC TCC AAC TAC GGA AAG TGC ACT GAC ATC TTT GCC CCG      1519
Arg Ala Tyr Phe Ser Asn Tyr Gly Lys Cys Thr Asp Ile Phe Ala Pro
            345                 350                 355

GGT CCC AAC ATT CTT TCC ACC TGG ACT GGC AGC AAG CAC GCT GTC AAC      1567
Gly Pro Asn Ile Leu Ser Thr Trp Thr Gly Ser Lys His Ala Val Asn
        360                 365                 370

ACC ATC TCT GGA ACC TCT ATG GCT TCT CCT CAC ATT GCT GGT CTG CTG      1615
Thr Ile Ser Gly Thr Ser Met Ala Ser Pro His Ile Ala Gly Leu Leu
    375                 380                 385

GCC TAC TTC GTT TCT CTG CAG CCT GCT CAG GAC TCT GCT TTC GCT GTC      1663
Ala Tyr Phe Val Ser Leu Gln Pro Ala Gln Asp Ser Ala Phe Ala Val
390                 395                 400                 405

GAT GAG CTT ACT CCT GCC AAG CTC AAG AAG GAT ATC ATC TCC ATC GCC      1711
Asp Glu Leu Thr Pro Ala Lys Leu Lys Lys Asp Ile Ile Ser Ile Ala
                410                 415                 420

ACC CAG GGT GCC CTT ACT GAT ATC CCA TCT GAC ACC CCC AAC              1753
Thr Gln Gly Ala Leu Thr Asp Ile Pro Ser Asp Thr Pro Asn
            425                 430                 435

GTAAGTTATA TTATCCATTT TGGTATAATG AAACAGAAAG TGGCTAACTG TTTTATTCTA    1813

G CTT CTC GCC TGG AAC GGC GGT GGT GCC GAC AAC TAC ACC CAG ATT         1859
  Leu Leu Ala Trp Asn Gly Gly Gly Ala Asp Asn Tyr Thr Gln Ile
                440                 445                 450

GTC GCC AAG GGT GGA TAC AAG GCC GGC AGT GAC AAC CTT AAG GAC CGC      1907
Val Ala Lys Gly Gly Tyr Lys Ala Gly Ser Asp Asn Leu Lys Asp Arg
                455                 460                 465
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TTT GAC GGA CTA GTC AAC AAG GCC GAG AAG TTG CTC GCT GAG GAG CTT      1955
Phe Asp Gly Leu Val Asn Lys Ala Glu Lys Leu Leu Ala Glu Glu Leu
            470                 475                 480

GGA GCT ATT TAC AGT GAG ATC CAG GGT GCT GTT GTT GCA TAG              1997
Gly Ala Ile Tyr Ser Glu Ile Gln Gly Ala Val Val Ala  *
       485                 490                 495

ATGCAAGACA AGACTTGATT TAGAGTGACG TAACTAGTTT CGTTTATGGC AGGGTATGGG    2057

AATTGGCTAA CCGAACACTG GCGCTGGTAT TTGTTTTTGC TGCTGCTTTT TGGTAACACG    2117

GAGAAGCCGA TGCATTGACT GCATTGGGTA CATTATCCTG ACATGGTTTA CCTGGTCTTT    2177

CATTATTATT ATAGCATACA TGTCCACAAC AATCTTTGAC ATCCTATCTA GAGATACATG    2237

TGCTTGCTTT TAACAGACTG CCAAATCAAT TATGCGACTG TTCTGCACAG ATAATCGTGG    2297

CTTGGTTTGA AGGCTGCCAT AAAGTCTAAC GCTGGCTACC AATTAGGTAG GAGTGTCCCC    2357

TTCCTGCCAG GTTGCTCCAG TCGTAGAAGT AGACTGATAT ATTGAAGATT GCCCATATAC    2417

CATGGACGCT CGTCTTATTC TACATCATAT ATGTCACTCC TAGTGACCAT ATAGACATGC    2477

TAACCATTGC ACAACCCCCC ACAGGTTCAA TCCAACCCAT GACCCCCTCT CATCTTCTGT    2537

TGTATTTTCA GGTTCTAGAT TTGCATACAT ACTACCCATC ATCGGAAGAC GGGTGAGGAG    2597

GCAGATGACC CGACATTATA TTTATTAATT GCTTAGGATG TTTCAACAAC ATTAAAAGTA    2657

TATCAATAAG CTTTTCCAGT TTATATTTAC TACCTAAGAT TACGGCATAT AGTGTATTCT    2717

GTGTGCGTAA GAGGTCGCCC TTAAATGGAA ACAGTTCGCG GTTGGAGATA TATATTTGTA    2777

GTGTTCAGGC GGAACGAGTA AAAAAAAAAA AAAATGAGAA GCTGGTGATA TTAACTCCGA    2837

TGTTTATCTT ACATATACCA ATGGATGTAG TCTCATTATA ACGCTTTCTC TGTAGTTTGG    2897

TTGTCATAGA ACTGAATGAC AGGTAAGTGT GTATGTATGT ACAGTACGCA CGGGGGGCCA    2957

TGTGGTCAAC CACACCCAAT GGGCGGTCTT GTCACTTTCC GGACTGGAAA TGAAACGTTC    3017

CATGGAAGAA ATCTGGATGA TTACCTTGAG TACGAGAGAA CTATGGTTGC CGGTAATGGG    3077

TGATTGCCAC AATCATCAGT TCGGTTGAGG CGTTCAACAT CTACGGTACG TTCAGTCACA    3137

TGAATCTGGG AATTCGGGCC TGGTATGCTG GTTTTCGCAA GAGATCCACC CGGCGTGTGC    3197

CAGGTATGCT ACATTTTCTC AGTCGAC                                        3224
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(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 496 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Arg Gly Ile Leu Gly Leu Ser Leu Leu Pro Leu Leu Ala Ala Ala
1 5 10 15

Ser Pro Val Ala Val Asp Ser Ile His Asn Gly Ala Ala Pro Ile Leu
20 25 30

Ser Ala Ser Asn Ala Lys Glu Val Pro Asp Ser Tyr Ile Val Val Phe
35 40 45

Lys Lys His Val Ser Ala Glu Thr Ala Ala Ala His His Thr Trp Val
50 55 60

Gln Asp Ile His Asp Ser Met Thr Gly Arg Ile Asp Leu Lys Lys Arg
65 70 75 80

Ser Leu Phe Gly Phe Ser Asp Asp Leu Tyr Leu Gly Leu Lys Asn Thr
85 90 95

Phe Asp Ile Ala Gly Ser Leu Ala Gly Tyr Ser Gly His Phe His Glu
100 105 110

Asp Val Ile Glu Gln Val Arg Arg His Pro Asp Val Glu Tyr Ile Glu
115 120 125

Lys Asp Thr Glu Val His Thr Met Glu Glu Thr Thr Glu Lys Asn Ala
130 135 140

Pro Trp Gly Leu Ala Arg Ile Ser His Arg Asp Ser Leu Ser Phe Gly
145 150 155 160

Thr Phe Asn Lys Tyr Leu Tyr Ala Ser Glu Gly Gly Glu Gly Val Asp
165 170 175

Ala Tyr Thr Ile Asp Thr Gly Ile Asn Ile Glu His Val Asp Phe Glu
180 185 190

Asp Arg Ala His Trp Gly Lys Thr Ile Pro Ser Asn Asp Glu Asp Ala
195 200 205

Asp Gly Asn Gly His Gly Thr His Cys Ser Gly Thr Ile Ala Gly Lys
210 215 220

Lys Tyr Gly Val Ala Lys Lys Ala Asn Ile Tyr Ala Val Lys Val Leu
225 230 235 240

Arg Ser Ser Gly Ser Gly Thr Met Ser Asp Val Val Leu Gly Val Glu
245 250 255

Trp Ala Val Gln Ser His Leu Lys Lys Ala Lys Asp Ala Lys Asp Ala
260 265 270

Lys Val Lys Gly Phe Lys Gly Ser Val Ala Asn Met Ser Leu Gly Gly
275 280 285

Ala Lys Ser Arg Thr Leu Glu Ala Ala Val Asn Ala Gly Val Glu Ala
290 295 300

Gly Leu His Phe Ala Val Ala Ala Gly Asn Asp Asn Ala Asp Ala Cys
305 310 315 320

Asn Tyr Ser Pro Ala Ala Ala Glu Asn Ala Ile Thr Val Gly Ala Ser
325 330 335

Thr Leu Gln Asp Glu Arg Ala Tyr Phe Ser Asn Tyr Gly Lys Cys Thr
340 345 350

Asp Ile Phe Ala Pro Gly Pro Asn Ile Leu Ser Thr Trp Thr Gly Ser
355 360 365

Lys His Ala Val Asn Thr Ile Ser Gly Thr Ser Met Ala Ser Pro His
370 375 380

Ile Ala Gly Leu Leu Ala Tyr Phe Val Ser Leu Gln Pro Ala Gln Asp
385 390 395 400

Ser Ala Phe Ala Val Asp Glu Leu Thr Pro Ala Lys Leu Lys Lys Asp
405 410 415

Ile Ile Ser Ile Ala Thr Gln Gly Ala Leu Thr Asp Ile Pro Ser Asp
420 425 430

Thr Pro Asn Leu Leu Ala Trp Asn Gly Gly Gly Ala Asp Asn Tyr Thr
435 440 445

Gln Ile Val Ala Lys Gly Gly Tyr Lys Ala Gly Ser Asp Asn Leu Lys
450 455 460

Asp Arg Phe Asp Gly Leu Val Asn Lys Ala Glu Lys Leu Leu Ala Glu
465 470 475 480

Glu Leu Gly Ala Ile Tyr Ser Glu Ile Gln Gly Ala Val Val Ala *
485 490 495

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5643 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus oryzae
(B) STRAIN: IFO4177
(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2701..2769
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: join(2282..2700, 2770..4949)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

AAGCTTCGTC CTCGCATCTC GGCCGGGTGA GTAAGGTATG GTATTATTCA TGAAGGGATC 60

TCGTTGGTTA CCGTTGTCTA TCCCTAAACA AAGGATTCAA GAGAACAACT CGGAATGCTC 120

CCTCCGCTTA AACCCCTTGA CTCACTGATG GTGTATGTAC TATGGGTACG ACGTTCGGGA 180

TGTGGACTAC CAACCAGAGA GTGATTAGAG AGTCCGGGTT CTCAGTCCAT GATTTTTGCA 240

TCTTTGAAAC AGACGATGCG GAGCGGTCAT TGGCGGAGTT TACTCCCAAA TACGGCCGAA 300

CGGGGTACTT TAAGTGGAAT CTCCGATTTT GGATCTAAGC TCATGAAGGA AAAGTACTAC 360

TAATGCGTAC CTGTGCCTAA TGTTAGTGCT AGTTCGTCTG TTGCATTTTA CCCGTCGGTT 420

AAGACGAATG GATCCGTTCA GGTTTTAAAA TAACTATCTA TGAAATATTT TAGATTTCCC 480

GACATAGTGG TTGGGATGTC TCGATTAACA CTAGGTACAT CAGGCTCAAT TGATTTTGGT 540

TTTAACGAAA CATGATATAG GTCAGGGTCG TGGACCACCC TCCGCCAGGG ATCAGGGGAC 600

GGTTACATGC GAAGGATTCT GATTATATTC ATGATTATGT CAAGCCTTTT CTCTCGTGTG 660

AAGAGGAGCA GAGAATCCGT ACGGGTTTAA TTTAATTTAG CGCCCTGCAG CTTCGAGAAC 720

ATCCCCAGCA ACGTTAAAAA CCACGAGCTA AAATGGGTCG CCACCGGAAG CACTCGAGTC 780

GAGAGATCGG TCGGCTCAGT ATTCGTAATA CCTGCGTTCC AGACGGTTTT GGTCGTTGGT 840

TTCACTCAGG GAACTTAATT CCAGCGGGAC CCAATATAAT TTGAATGATT CATGATACAT 900

CCATTCGTTT GAACCGATCC TGCAAGAGTT CTGTCTGATT TGGTCAACAT AGTTTTCCTC 960

TGGGGGAGAC TGGGGAAGAG TCAACACAAT GGTCAGGGAG AGAAGAATGA AAGCTCTCGC 1020

AAGTGGATGA TCATGCTACG TACTGTAGGA ATAAAATTAA TTAATGCGAG GCTGCAAGTA 1080

TCCCTGCGCC GATTTTCTCT TCTTACGGCG GGAACCAAAA AATGTGACGC TGTGATTTTC 1140

TGGAAAAGGT AAGGATGTTT AGTTTCCCAG GATTATTACT GGTTCCGTAT GTGTATGTGT 1200

ATGGATATCA TTCCGTATGG ATACGCCCGT TTCCTCCGCC CAGAACCAGT CCGTCATCCA 1260

TCCTCCACTC TTTCTTCTCT TAGAGCCTTT CCACCTCTCT TCACTTTCTT TTTCTTTCCC 1320

CCCTCCCTCT TTGCTTTCCC TCTCCCAGTA TTATTCTTAT ATTATCGGTT TGACCGTCGC 1380

CTCAGTATCG GCCCCCCGTG AATCACTTTT CGTTTCTCTT GTATTTTACT TTCCTATCTG 1440

GGATTGCTCC TCGATTAGCA GCTCTACTTC ATTCGGCCAT GTGCGTCTAG AGGGTCTAGC 1500

CCCTCTCTCT CTTTGCACTG ACTGTCAGCC ATACCATAGT ATCATCCCGG AATTAAGAAA 1560

AAAAAAGAAA TTATTCTACC TCCGATCTGG ACAAATTATA ACCAGGAGAA AATCAAGCGA 1620

AAGAGGGGCA AAGGAGGAGA CACCATTAAA ACTGGGTCTG GTTTGATTCA TGACATACAT 1680

TCGTCGTCTT GAATTTCAAT AGGTACGGAC TGATGCATTC CACTCGAGCC TTTTTAGCTG 1740

CGTGTCCGTC TCCAATCGCA CTTCTTTTCT TATTTCCTTG TGGGATAAAT TGATTATTTA 1800

CCGTTTCGTT TTCTCTATAT TGCGGTGGTG GTGCGACCCA TCCAACTATT ATTATTATAA 1860

TTGGAATTTG ATTTGGATTT TGATTCCTGT GACGGATCTC AGACCAAGTG CCTAAACTAT 1920

AACTGACTTG GACCCCCTTC AGATCCTAGC TTCCCGATTC TTTTCCACCA CTGCTGCATC 1980

CTCTTCCTGC ACGCAGCGTT CGTTTAGGGC GGGTAGACTG GAATTTATTC CTTGCGCCAC 2040

GGACCAATCG CTCCCTCGAC GCTCTCATTC CTGCGTCGAG CTCTTTTTCC CTCGACTCTC 2100

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ATTGCTTGCT GGGCTGGTTC TTGAACCTCT TCAATCGTCC TTATCTCTTT CCCCCCATCC     2160

GGCCTGTGAT TCCTATCTTT CCTTTTTTTC TTCCCTTTCT TGTTTGATCC CCCCTCCTCC     2220

CCGTCTTATC GCCTACTATC GTGATCCCCG CCCTTCCCAA TAAAGAGTAG GGCGTGTGAA     2280

C ATG TCC GGG TTA ACC CTC GGG CGA GGC CCT GGG GGC GTG CGA CCG          2326
  Met Ser Gly Leu Thr Leu Gly Arg Gly Pro Gly Gly Val Arg Pro
    1               5                  10                  15

ACT CAA ACC GCA ACT TTT ACC ACC CAC CAC CCG TCC GCC GAT GCT GAC       2374
Thr Gln Thr Ala Thr Phe Thr Thr His His Pro Ser Ala Asp Ala Asp
                20                  25                  30

CGC TCC TCC AAC AAC CTC CCC CCT ACC TCC TCG CAG CTG TCC GAT GAC       2422
Arg Ser Ser Asn Asn Leu Pro Pro Thr Ser Ser Gln Leu Ser Asp Asp
            35                  40                  45

TTT TCT TTC GGT TCC CCT CTG AGC CCC GCC GAC TCA CAG GCC CAT GAC       2470
Phe Ser Phe Gly Ser Pro Leu Ser Pro Ala Asp Ser Gln Ala His Asp
        50                  55                  60

GGC CTA CTT CAG GAC TCC CTC TTC CCT GAA TGG GGG TCT GGT GCG CCT       2518
Gly Leu Leu Gln Asp Ser Leu Phe Pro Glu Trp Gly Ser Gly Ala Pro
    65                  70                  75

CGA CCC GGC ATT GAC AGT CCG GAT GAG ATG CAG AGG CAA GAT CCG CTA       2566
Arg Pro Gly Ile Asp Ser Pro Asp Glu Met Gln Arg Gln Asp Pro Leu
80                  85                  90                  95

GCG ACT CAA ATA TGG AAG CTC TAT TCT AGG ACC AAG GCC CAG TTG CCC       2614
Ala Thr Gln Ile Trp Lys Leu Tyr Ser Arg Thr Lys Ala Gln Leu Pro
                100                 105                 110

AAC CAG GAG CGT ATG GAA AAC CTG ACC TGG CGG ATG ATG GCG ATG AGT       2662
Asn Gln Glu Arg Met Glu Asn Leu Thr Trp Arg Met Met Ala Met Ser
            115                 120                 125

TTG AAA CGT AAG GAG CGG GAA CGT GCT CAA CAG TCC  AT  GTAGGTGTTC       2710
Leu Lys Arg Lys Glu Arg Glu Arg Ala Gln Gln Ser  Met
        130                 135                 140

TCCCTCTGTA GAGGAACGGC TGGACCCGCT CATCATTAAT TTTTTTTTTG TCTGTGAAG G   2770

TTT CCT GCG AGA CGC GGT AGC GCT GGC CCC AGT GGT ATC GCT CAA CTG       2818
Phe Pro Ala Arg Arg Gly Ser Ala Gly Pro Ser Gly Ile Ala Gln Leu
                145                 150                 155

CGC ATT TCC GAC CCG CCC GTT GCC ACC GGT AAC CCT CAG TCA ACC GAC       2866
Arg Ile Ser Asp Pro Pro Val Ala Thr Gly Asn Pro Gln Ser Thr Asp
            160                 165                 170

CTG ACC GCC GAC CCT ATG AAC CTC GAC GAT TTC ATC GTG CCC TTC GAA       2914
Leu Thr Ala Asp Pro Met Asn Leu Asp Asp Phe Ile Val Pro Phe Glu
        175                 180                 185

TCT CCT TCG GAC CAC CCC TCG CCC AGT GCC GTC AAG ATT TCC GAC TCC       2962
Ser Pro Ser Asp His Pro Ser Pro Ser Ala Val Lys Ile Ser Asp Ser
    190                 195                 200

ACG GCG TCC GCG GCC ATT CCC ATC AAG TCC CGG AAA GAC CAG CTG AGA       3010
Thr Ala Ser Ala Ala Ile Pro Ile Lys Ser Arg Lys Asp Gln Leu Arg
205                 210                 215                 220
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GAT TCT ACC CCG GTG CCG GCC TCG TTC CAC CAT CCG GCT CAG GAT CAA      3058
Asp Ser Thr Pro Val Pro Ala Ser Phe His His Pro Ala Gln Asp Gln
                225                 230                 235

CGG AAG AAC AGT GAA TTT GGC TAC GTC CCC CGT CGC GTG CGC AAG ACG      3106
Arg Lys Asn Ser Glu Phe Gly Tyr Val Pro Arg Arg Val Arg Lys Thr
            240                 245                 250

AGT ATC GAC GAG CGT CAA TTT TTC TCA CTG CAG GTG CCG ACC CGA AAG      3154
Ser Ile Asp Glu Arg Gln Phe Phe Ser Leu Gln Val Pro Thr Arg Lys
        255                 260                 265

CGA CCG GCC GAA TCC TCG CCC CAG GTA CCC CCC GTT TCC AAC TCG ATG      3202
Arg Pro Ala Glu Ser Ser Pro Gln Val Pro Pro Val Ser Asn Ser Met
    270                 275                 280

TTG GCC CAC GAT CCG GAC CTC GCT TCC GGC GTG CCC GAT TAT GCC TTG      3250
Leu Ala His Asp Pro Asp Leu Ala Ser Gly Val Pro Asp Tyr Ala Leu
285                 290                 295                 300

GAC GCC CCG TCC TCG GCC TTT GGC TTC CAT CAG GGT AAC CAC CAT CCG      3298
Asp Ala Pro Ser Ser Ala Phe Gly Phe His Gln Gly Asn His His Pro
                305                 310                 315

GTC AAT CAT CAC AAC CAC ACC TCC CCC GGG GCA CCG TTT GGC TTG GAT      3346
Val Asn His His Asn His Thr Ser Pro Gly Ala Pro Phe Gly Leu Asp
            320                 325                 330

ACG TTC GGC CTG GGA GAT GAT CCA ATC TTG CCC TCC GCG GGC CCC TAC      3394
Thr Phe Gly Leu Gly Asp Asp Pro Ile Leu Pro Ser Ala Gly Pro Tyr
        335                 340                 345

CAG TCG CAA TTC ACC TTC TCA CCC AGC GAG TCT CCG ATG GCC TCC GGT      3442
Gln Ser Gln Phe Thr Phe Ser Pro Ser Glu Ser Pro Met Ala Ser Gly
    350                 355                 360

CAT CCG TTT GCG AAC CTC TAT TCG CAT ACC CCG GTG GCT TCG TCC CTC      3490
His Pro Phe Ala Asn Leu Tyr Ser His Thr Pro Val Ala Ser Ser Leu
365                 370                 375                 380

AAC TCG ACG GAT TTC TTC TCT CCA CCG CCA TCA GGC TAC CAG TCC ACG      3538
Asn Ser Thr Asp Phe Phe Ser Pro Pro Pro Ser Gly Tyr Gln Ser Thr
                385                 390                 395

GCA TCC ACG CCG CAG CCC ACC TAC GAC GGG GAC CAT TCC GTT TAT TTC      3586
Ala Ser Thr Pro Gln Pro Thr Tyr Asp Gly Asp His Ser Val Tyr Phe
            400                 405                 410

GAT ATG CCG TCG GGC GAC GCG CGC ACC CAG CGC CGC ATT CCG AAC TAT      3634
Asp Met Pro Ser Gly Asp Ala Arg Thr Gln Arg Arg Ile Pro Asn Tyr
        415                 420                 425

ATT TCG CAT CGG TCC AAC TTG TCT GCT TCG CTG CAG CCT CGG TAT ATG      3682
Ile Ser His Arg Ser Asn Leu Ser Ala Ser Leu Gln Pro Arg Tyr Met
    430                 435                 440

TTC AAC CAG AAC AAC CAT GAA CAG GCC AGT TCG TCG ACG GTG CAT TCG      3730
Phe Asn Gln Asn Asn His Glu Gln Ala Ser Ser Ser Thr Val His Ser
445                 450                 455                 460

CCG AGC TAC CCC ATT CCC CAG CCG CAA CAT GTG GAC CCC ACT CAG GTG      3778
Pro Ser Tyr Pro Ile Pro Gln Pro Gln His Val Asp Pro Thr Gln Val
                465                 470                 475

TTG AAC GCC ACC AAT TAC TCG ACC GGC AAC TCC CAC CAT ACC GGC GCC      3826
Leu Asn Ala Thr Asn Tyr Ser Thr Gly Asn Ser His His Thr Gly Ala
            480                 485                 490
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ATG TTT TCA TTT GGA GCC GAT TCA GAT AAC GAG GAT GAC GAT GGT CAT      3874
Met Phe Ser Phe Gly Ala Asp Ser Asp Asn Glu Asp Asp Asp Gly His
        495                 500                 505

CAG CTG TCC GAG CGG GCT GGT CTG GCG ATG CCG ACT GAA TAT GGG GAC      3922
Gln Leu Ser Glu Arg Ala Gly Leu Ala Met Pro Thr Glu Tyr Gly Asp
    510                 515                 520

GAG GAC GGG TTC TCG TCG GGC ATG CAG TGG GAT GGG CAG TTC CCG GGC      3970
Glu Asp Gly Phe Ser Ser Gly Met Gln Trp Asp Gly Gln Phe Pro Gly
525                 530                 535                 540

TCC TTC CAT TCG CTG CCG GGC TTT GGC CCT CAA CAT CGC AAG CAT GTT      4018
Ser Phe His Ser Leu Pro Gly Phe Gly Pro Gln His Arg Lys His Val
                545                 550                 555

ACC ATC GGG TCC ACG GAC ATG ATG GAC ACC CCC GAG GAG TGG AAT CAC      4066
Thr Ile Gly Ser Thr Asp Met Met Asp Thr Pro Glu Glu Trp Asn His
            560                 565                 570

GGT GGC AGT TTG GGT CGG ACT CAT GGG TCG GTG GCT TCG GTC AGT GAG      4114
Gly Gly Ser Leu Gly Arg Thr His Gly Ser Val Ala Ser Val Ser Glu
        575                 580                 585

GTG CGC AAC CGA GAG CAG GAC CCT CGC CGG CAG AAG ATT GCC CGC ACC      4162
Val Arg Asn Arg Glu Gln Asp Pro Arg Arg Gln Lys Ile Ala Arg Thr
    590                 595                 600

ACG TCC ACC CCC AAT ACG GCC CAG CTG TTG CGC CAA AGC ATG CAC TCT      4210
Thr Ser Thr Pro Asn Thr Ala Gln Leu Leu Arg Gln Ser Met His Ser
605                 610                 615                 620

AAT AAC AAT ACG TCT CAT ACC TCC CCT AAT ACG CCG CCC GAG TCC GCC      4258
Asn Asn Asn Thr Ser His Thr Ser Pro Asn Thr Pro Pro Glu Ser Ala
                625                 630                 635

CTG AGC AGC GCA GTT CCG TCC CGC CCG GCC AGT CCC GGG GGC AGC AAG      4306
Leu Ser Ser Ala Val Pro Ser Arg Pro Ala Ser Pro Gly Gly Ser Lys
            640                 645                 650

AAC GGC GAC CAA GGC AGC AAC GGA CCG ACC ACC TGC ACG AAC TGC TTC      4354
Asn Gly Asp Gln Gly Ser Asn Gly Pro Thr Thr Cys Thr Asn Cys Phe
        655                 660                 665

ACT CAA ACC ACT CCG CTG TGG CGT CGG AAC CCA GAG GGC CAG CCA CTG      4402
Thr Gln Thr Thr Pro Leu Trp Arg Arg Asn Pro Glu Gly Gln Pro Leu
    670                 675                 680

TGC AAT GCC TGC GGG TTG TTT TTG AAA TTG CAC GGT GTC GTG CGC CCT      4450
Cys Asn Ala Cys Gly Leu Phe Leu Lys Leu His Gly Val Val Arg Pro
685                 690                 695                 700

CTG TCC CTG AAA ACG GAC GTT ATC AAA AAG CGC AAC CGT AGC AGT GCC      4498
Leu Ser Leu Lys Thr Asp Val Ile Lys Lys Arg Asn Arg Ser Ser Ala
                705                 710                 715

AAC AGC TTG GCG GTT GGG ACC TCC CGT GCG TCG AAG AAG ACA GCC CGC      4546
Asn Ser Leu Ala Val Gly Thr Ser Arg Ala Ser Lys Lys Thr Ala Arg
            720                 725                 730

AAG AAC TCG GTG CAG CAA GCA TCC GTC ACG ACT CCG ACA TCA AGC CGC      4594
Lys Asn Ser Val Gln Gln Ala Ser Val Thr Thr Pro Thr Ser Ser Arg
        735                 740                 745
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GCT CAG AAT GGG ACT TCC TTC GAA TCC CCG CCC GCC GGC TTT AGT GCT      4642
Ala Gln Asn Gly Thr Ser Phe Glu Ser Pro Pro Ala Gly Phe Ser Ala
    750                 755                 760

GCC GCG GGA CGG TCG AAT GGG GTG GTA CCC ATT GCC GCC GCT CCT CCG      4690
Ala Ala Gly Arg Ser Asn Gly Val Val Pro Ile Ala Ala Ala Pro Pro
765                 770                 775                 780

AAG GCA GCT CCC TCC GCA GCC GCC TCC CCT AGC ACG GGC CAG ACC CGC      4738
Lys Ala Ala Pro Ser Ala Ala Ala Ser Pro Ser Thr Gly Gln Thr Arg
                785                 790                 795

AAC CCG ATC CAG GCT GCC CCG AAA CGT CAA CGA CGG CTG GAA AAG GCC      4786
Asn Pro Ile Gln Ala Ala Pro Lys Arg Gln Arg Arg Leu Glu Lys Ala
            800                 805                 810

ACG GAG ATG GAA ACG GAC GAG GCT AAC AAG TCC GCG GGA GGC CGA TCC      4834
Thr Glu Met Glu Thr Asp Glu Ala Asn Lys Ser Ala Gly Gly Arg Ser
        815                 820                 825

AAG GTG GTG CCT CTG GCA CCC GCC ATG CCA CCG GCA GCA GCC AAT CCG      4882
Lys Val Val Pro Leu Ala Pro Ala Met Pro Pro Ala Ala Ala Asn Pro
    830                 835                 840

GCG AAC CAT AGT ATT GCC GGA GGC CAA GGG GCT AGT CAG GAA TGG GAG      4930
Ala Asn His Ser Ile Ala Gly Gly Gln Gly Ala Ser Gln Glu Trp Glu
845                 850                 855                 860

TGG TTG ACG ATG AGT CTGTAATGGC CGCGCTTACC TCTCTACTTC TCTACACTCG      4985
Trp Leu Thr Met Ser Leu
                865

TTTCTTAATA TCTTTCTTGA ACCCCCCCTT ATATTTTCCC ACCGTTGATG CTACGCCATG    5045

ACCGATAGAG ATGATGAATA CTGCAACCAA TGGAATCTCG CTAGACGAGA GGTGTTAGAT    5105

GACGTGGCCC GCGATGCACT TAATGAGATA CGAGGAGGTG CAATGCGTTG GTTACGCTAG    5165

TTTAATGGTA ACATGACGAG GGATATTCGC TCTGTTATTT CGGGCTTTGA TCTGTTTCAG    5225

TCTGCGATTT AACAGCGACT GATCCTCTGC TGTGACAATA CACAGCTTGT CTTGTGGTTC    5285

TGTTGTGGCT TTCTGTTTGT TTGGCTGATT TGATTTATGC TTGATACAAT CGCGTCTGTC    5345

CGGACCCCGG CCTTTGTTTT GTTTTCAGTT CTGATTCTTC ACTGTTTCTG ATTCTCTTGT    5405

TCATGTTTTT GATTTGTTCA AGGCTTGGGG CCGGGCAGAA GTGCGCATCT CTGCTTTGTG    5465

TTTTCCGTCA CCGTGCATAG ACGCTGTATG TATATGCTAC AGCAAGATTC TACTTATCCA    5525

GTCTGAGCCT GTATTCATTG AAGTGTAGCC AGCTGTCGAA TGAGCTTTTT AACGATATTG    5585

TTTTGTTGAG TAGTCAACAA GTAGTATCTG TATATTCCGG AGTCTAAGTA AGACACTT      5643
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(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 866 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Ser Gly Leu Thr Leu Gly Arg Gly Pro Gly Gly Val Arg Pro Thr
1 5 10 15

Gln Thr Ala Thr Phe Thr Thr His His Pro Ser Ala Asp Ala Asp Arg
20 25 30

Ser Ser Asn Asn Leu Pro Pro Thr Ser Ser Gln Leu Ser Asp Asp Phe
35 40 45

Ser Phe Gly Ser Pro Leu Ser Pro Ala Asp Ser Gln Ala His Asp Gly
50 55 60

Leu Leu Gln Asp Ser Leu Phe Pro Glu Trp Gly Ser Gly Ala Pro Arg
65 70 75 80

Pro Gly Ile Asp Ser Pro Asp Glu Met Gln Arg Gln Asp Pro Leu Ala
85 90 95

Thr Gln Ile Trp Lys Leu Tyr Ser Arg Thr Lys Ala Gln Leu Pro Asn
100 105 110

Gln Glu Arg Met Glu Asn Leu Thr Trp Arg Met Met Ala Met Ser Leu
115 120 125

Lys Arg Lys Glu Arg Glu Arg Ala Gln Gln Ser Met Phe Pro Ala Arg
130 135 140

Arg Gly Ser Ala Gly Pro Ser Gly Ile Ala Gln Leu Arg Ile Ser Asp
145 150 155 160

Pro Pro Val Ala Thr Gly Asn Pro Gln Ser Thr Asp Leu Thr Ala Asp
165 170 175

Pro Met Asn Leu Asp Asp Phe Ile Val Pro Phe Glu Ser Pro Ser Asp
180 185 190

His Pro Ser Pro Ser Ala Val Lys Ile Ser Asp Ser Thr Ala Ser Ala
195 200 205

Ala Ile Pro Ile Lys Ser Arg Lys Asp Gln Leu Arg Asp Ser Thr Pro
210 215 220

Val Pro Ala Ser Phe His His Pro Ala Gln Asp Gln Arg Lys Asn Ser
225 230 235 240

Glu Phe Gly Tyr Val Pro Arg Arg Val Arg Lys Thr Ser Ile Asp Glu
245 250 255

Arg Gln Phe Phe Ser Leu Gln Val Pro Thr Arg Lys Arg Pro Ala Glu
260 265 270

Ser Ser Pro Gln Val Pro Pro Val Ser Asn Ser Met Leu Ala His Asp
275 280 285

Pro Asp Leu Ala Ser Gly Val Pro Asp Tyr Ala Leu Asp Ala Pro Ser
290 295 300

Ser Ala Phe Gly Phe His Gln Gly Asn His His Pro Val Asn His His
305 310 315 320

Asn His Thr Ser Pro Gly Ala Pro Phe Gly Leu Asp Thr Phe Gly Leu
325 330 335

Gly Asp Asp Pro Ile Leu Pro Ser Ala Gly Pro Tyr Gln Ser Gln Phe
340 345 350

Thr Phe Ser Pro Ser Glu Ser Pro Met Ala Ser Gly His Pro Phe Ala
355 360 365

Asn Leu Tyr Ser His Thr Pro Val Ala Ser Ser Leu Asn Ser Thr Asp
370 375 380

Phe Phe Ser Pro Pro Pro Ser Gly Tyr Gln Ser Thr Ala Ser Thr Pro
385 390 395 400

Gln Pro Thr Tyr Asp Gly Asp His Ser Val Tyr Phe Asp Met Pro Ser
405 410 415

Gly Asp Ala Arg Thr Gln Arg Arg Ile Pro Asn Tyr Ile Ser His Arg
420 425 430

Ser Asn Leu Ser Ala Ser Leu Gln Pro Arg Tyr Met Phe Asn Gln Asn
435 440 445

Asn His Glu Gln Ala Ser Ser Ser Thr Val His Ser Pro Ser Tyr Pro
450 455 460

Ile Pro Gln Pro Gln His Val Asp Pro Thr Gln Val Leu Asn Ala Thr
465 470 475 480

Asn Tyr Ser Thr Gly Asn Ser His His Thr Gly Ala Met Phe Ser Phe
485 490 495

Gly Ala Asp Ser Asp Asn Glu Asp Asp Asp Gly His Gln Leu Ser Glu
500 505 510

Arg Ala Gly Leu Ala Met Pro Thr Glu Tyr Gly Asp Glu Asp Gly Phe
515 520 525

Ser Ser Gly Met Gln Trp Asp Gly Gln Phe Pro Gly Ser Phe His Ser
530 535 540

Leu Pro Gly Phe Gly Pro Gln His Arg Lys His Val Thr Ile Gly Ser
545 550 555 560

Thr Asp Met Met Asp Thr Pro Glu Glu Trp Asn His Gly Gly Ser Leu
565 570 575

Gly Arg Thr His Gly Ser Val Ala Ser Val Ser Glu Val Arg Asn Arg
580 585 590

Glu Gln Asp Pro Arg Arg Gln Lys Ile Ala Arg Thr Thr Ser Thr Pro
595 600 605

Asn Thr Ala Gln Leu Leu Arg Gln Ser Met His Ser Asn Asn Asn Thr
610 615 620

Ser His Thr Ser Pro Asn Thr Pro Pro Glu Ser Ala Leu Ser Ser Ala
625 630 635 640

Val Pro Ser Arg Pro Ala Ser Pro Gly Gly Ser Lys Asn Gly Asp Gln
645 650 655

Gly Ser Asn Gly Pro Thr Thr Cys Thr Asn Cys Phe Thr Gln Thr Thr
660 665 670

Pro Leu Trp Arg Arg Asn Pro Glu Gly Gln Pro Leu Cys Asn Ala Cys
675 680 685

Gly Leu Phe Leu Lys Leu His Gly Val Val Arg Pro Leu Ser Leu Lys
690 695 700

Thr Asp Val Ile Lys Lys Arg Asn Arg Ser Ser Ala Asn Ser Leu Ala
705 710 715 720

Val Gly Thr Ser Arg Ala Ser Lys Lys Thr Ala Arg Lys Asn Ser Val
725 730 735

Gln Gln Ala Ser Val Thr Thr Pro Thr Ser Ser Arg Ala Gln Asn Gly
740 745 750

Thr Ser Phe Glu Ser Pro Pro Ala Gly Phe Ser Ala Ala Ala Gly Arg
755 760 765

Ser Asn Gly Val Val Pro Ile Ala Ala Ala Pro Pro Lys Ala Ala Pro
770 775 780

Ser Ala Ala Ala Ser Pro Ser Thr Gly Gln Thr Arg Asn Pro Ile Gln
785 790 795 800

Ala Ala Pro Lys Arg Gln Arg Arg Leu Glu Lys Ala Thr Glu Met Glu
805 810 815

Thr Asp Glu Ala Asn Lys Ser Ala Gly Gly Arg Ser Lys Val Val Pro
820 825 830

Leu Ala Pro Ala Met Pro Pro Ala Ala Ala Asn Pro Ala Asn His Ser
835 840 845

Ile Ala Gly Gly Gln Gly Ala Ser Gln Glu Trp Glu Trp Leu Thr Met
850 855 860

Ser Leu
865

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 27 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Primer 19819

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7

GAAGATCTGC GCGGATGTAC ATTGTAG 27

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Primer 19821

(iii) HYPOTHETICAL: YES
(iii) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8

TTAGTCAGAA ATTCGTCCCG 20

(2) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA Primer 19820
(iii) HYPOTHETICAL: YES
(iii) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9

CCCAAGCTTC ATGCTCGACC AGGGCCTCCT 30

(2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA Primer 19818
(iii) HYPOTHETICAL: YES
(iii) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10

GGTCTGTGTT AACCAAAGAA C 21

(2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 nucleotides
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA Primer 8681
(iii) HYPOTHETICAL: YES
(iii) ANTI-SENSE: NO
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11

GATCCACCAT GAAG 14

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 14 nucleotides

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA Primer 8747

(iii) HYPOTHETICAL: YES

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12

GTGGTACTTC AGCT 14

PATENT CLAIMS

1. A fungus, wherein the *areA* gene by recombinant DNA technology has been modified in a way by which it cannot be expressed in a way providing for a functional AreA activator, and wherein the genes encoding for the extracellular proteases PepC and/or PepE have been inactivated in a manner whereby they are not expressed to produce functional proteases.

2. The fungus of claim 1, wherein said inactivation has been obtained by deletion of all or parts of the *areA*, *pepC*, and/or *pepE* genes.

3. The fungus of claim 1, wherein said inactivation has been obtained by deletion of all or parts of the *areA*, and *pepE* genes.

4. The fungus of claim 1, wherein said inactivation has been obtained by interfering with the regulation of the expression signals regulating the expression of the *areA*, *pepC*, and/or *pepE* genes themselves.

5. The fungus of claim 1, wherein said inactivation has been obtained by interfering with the regulation of the expression signals regulating the expression of the *areA*, and *pepE* genes themselves.

6. The fungus of claim 1, wherein said inactivation has been obtained by using anti-sense technology.

7. The fungus of claim 1, wherein said inactivation has been obtained by inserting extra DNA internally in the *areA*, *pepC*, and/or *pepE* genes.

8. The fungus of any of claims 1 to 7, being a filamentous fungus, preferably belonging to a genus selected from the group comprising *Aspergillus*, *Trichoderma*, *Humicola*, *Candida*, *Acremonium*, *Fusarium*, and *Penicillium*

9. The fungus of claim 8, which belongs to a species selected from the group comprising *A. oryzae*, *A. niger*, *A. awamori*, *A. phoenicis*, *A. japonicus*, *A. foetidus*, *A. nidulans*, *T. reesei*, *T. harzianum*, *H. insolens*, *H. lanuginosa*, *F. graminearum*, *F. solani*, *P. chrysogenum*, and others.

10. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by deletion of the *areA*, *pepC*, and/or *pepE* genes, which method comprises

i) cloning of the *areA*, *pepC*, and/or *pepE* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *areA* gene, the *pepC* gene, and/or the *pepE* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *areA*$^-$, *pepC*$^-$, and/or *pepE*$^-$.

11. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by deletion of the *areA* and *pepE* genes, which method comprises

i) cloning of the *areA* and *pepE* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *areA* gene and the *pepE* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *areA*$^-$, and *pepE*$^-$.

12. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by deletion of the *areA* and *pepC* genes, which method comprises

i) cloning of the *areA* and *pepC* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *areA* gene and the *pepC* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *areA*$^-$, and *pepC*$^-$.

13. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by deletion of the *pepC* and *pepE* genes, which method comprises

i) cloning of the *pepC* and *pepE* genes from a fungus of interest,

ii) producing DNA constructs each comprising one among the *pepC* gene and the *pepE* gene, wherein an internal part has been substituted, deleted, or extra DNA has been inserted,

iii) transforming said fungus with the constructs, and

iv) isolating transformants which are *pepC*$^-$, and *pepE*$^-$.

14. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by using anti-sense technology, which method comprises

i) construction of expression plasmids, each of which give rise to synthesis of an RNA molecule complementary to the mRNA transcribed from the *areA* gene, the *pepC* gene, and/or the *pepE* gene,

ii) transformation of the host fungus with said expression plasmids and a suitable marker, either on separate plasmids or on the same plasmid,

iii) selection of transformants using said marker, and

iv) screening selected transformants for strains exhibiting a reduction in the synthesis of the AreA, PepC, and/or PepE products.

15. A method for producing a fungus according to claim 1, wherein said inactivation has been obtained by using anti-sense technology, which method comprises

i) construction of expression plasmids, each of which give rise to synthesis of an RNA molecule complementary to the mRNA transcribed from the *areA* gene and the *pepE* gene,

ii) transformation of the host fungus with said expression plasmids and a suitable marker, either on separate plasmids or on the same plasmid,

iii) selection of transformants using said marker, and

iv) screening selected transformants for strains exhibiting a reduction in the synthesis of the AreA, PepC, and PepE products.

16. A process for the production of a desired gene product, whereby a fungus according to any of the claims 1 to 9 is cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

17. A process for the production of a desired gene product, whereby a fungus according to any of the claims 1 to 9, which has been transformed to integrate a DNA sequence coding for the desired gene product into the genome of the fungus in a functional manner, is cultivated in a suitable growth medium at appropriate conditions and the desired gene product is recovered and purified.

18. A process for producing a desired polypeptide comprising cultivating a fungus in an appropriate growth medium and recovering said polypeptide from said culture, said fungus carrying a recombinant DNA construct capable of causing

expression of said polypeptide or a precursor thereof in said fungus, said fungus further being characterized by producing lower amounts of functional AreA, PepC, and/or PepE than the wild-type of said fungus.

19. A method according to claim 18, wherein said fungus has been modified to produce lower than wild-type amounts of AreA, PepC, and/or PepE by a process comprising transforming a parent of said fungus with DNA constructs capable of causing reduced production of functional AreA, PepC, and/or PepE when integrated in the genome of said fungus.

20. A method according to claim 18, wherein said polypeptide is secreted to the extracellular medium by said fungus.

21. A method according to claim 18, wherein said fungus produces higher amounts of said polypeptide than a similar fungus where said similar fungus produces AreA, PepC, and/or PepE in amounts similar to those produced by the wild-type of said fungus, said similar fungus being identical to said fungus in all other respects.

22. The process of claim 14 or 15 to 21, wherein said gene product is a secreted protein.

23. The process of any of the claims 14 to 20, wherein said desired gene product is an industrial peptide or protein, preferably an enzyme.

24. The process of claim 23, wherein said enzyme is selected from the group comprising a protease, lipase, cutinase, cellulase, chymosin.

25. The process of any of the claims 15 to 22, wherein said desired gene product is a therapeutically active peptide or protein.

26. The process of claim 25, wherein said therapeutically active peptide or protein is selected from the group comprising insulin, growth hormone, glucagon, somatostatin, interferon, PDGF, factor VII, factor VIII, urokinase, tPA, EPO, or TPO.

27. A gene product produced in accordance with any of the processes 15 to 26.

28. A DNA sequence coding for the *pepC* gene from *A. oryzae* (SEQ ID No. 1) or functional alleles thereof.

29. A PepC protease from *A. oryzae* (SEQ ID No. 2).

30. A process for the production of the PepC protease of claim 29 comprising transforming a suitable host with a DNA construct comprising a DNA sequence of claim 28, selecting a transformant capable of producing said PepC protease, cultivating said transformant in an appropriate growth medium and recovering said PepC protease from said culture.

31 A DNA sequence coding for the *pepE* gene from *A. oryzae* (SEQ ID No. 3) or functional alleles thereof.

32. A PepE protease from *A. oryzae* (SEQ ID No. 4).

33. A process for the production of the PepE protease of claim 32 comprising transforming a suitable host with a DNA construct comprising a DNA sequence of claim 31, selecting a transformant capable of producing said PepE protease, cultivating said transformant in an appropriate growth medium and recovering said PepE protease from said culture.

34. The process of claim 30 or 33, wherein said host is a fungus, according to any of the claims 1 to 9.

35. The process of claim 30, 33 or 34, wherein said host is *A. oryzae*, and wherein said DNA construct comprising a DNA sequence of claim 28 or 31, provides for an extra copy of the gene encoding said PepC or PepE protease.

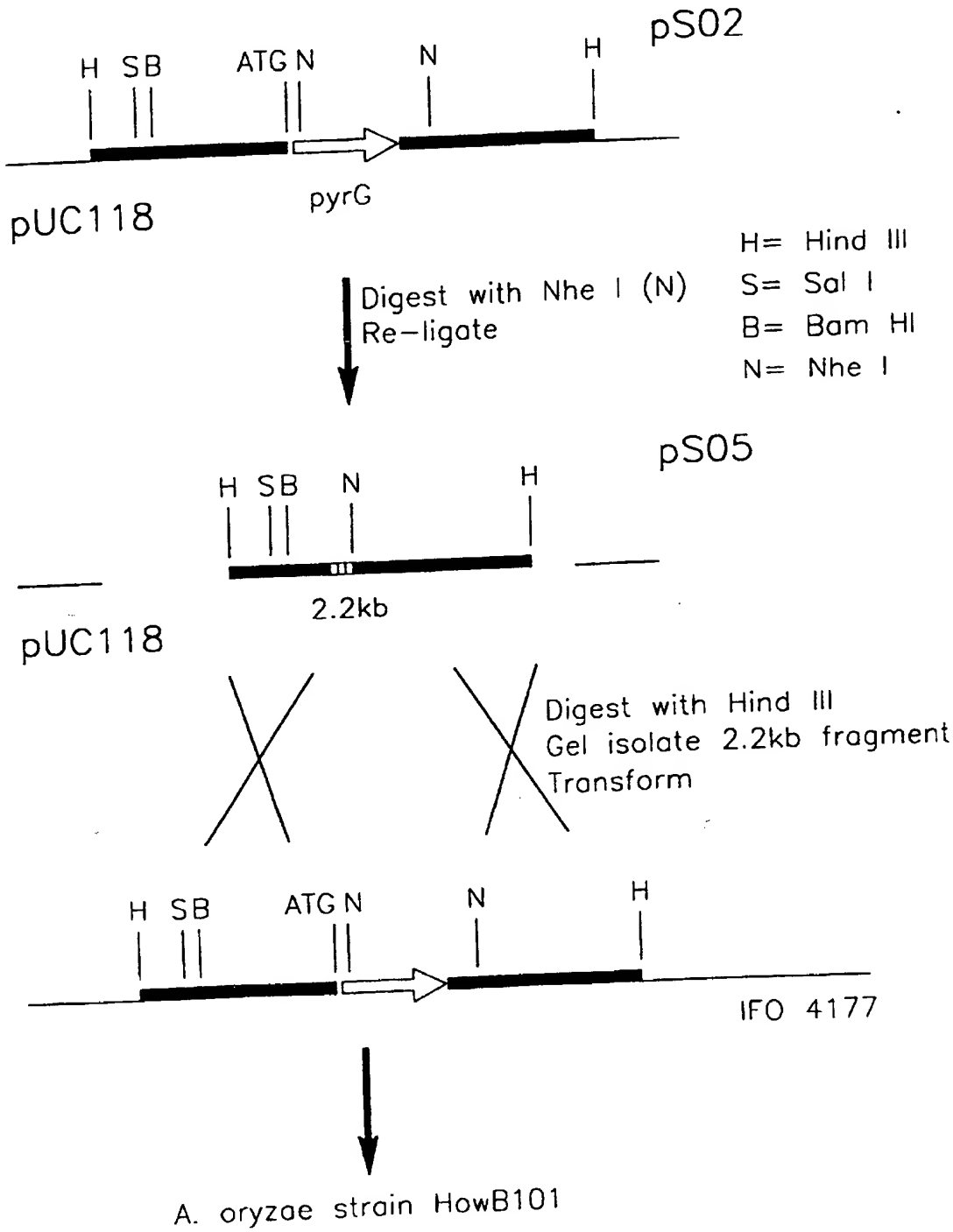

pS02
H SB ATG N N H
pyrG
pUC118
H= Hind III
S= Sal I
B= Bam HI
N= Nhe I
Digest with Nhe I (N)
Re-ligate
pS05
H SB N H
2.2kb
pUC118
Digest with Hind III
Gel isolate 2.2kb fragment
Transform
H SB ATG N N H
IFO 4177
A. oryzae strain HowB101

FIG. 1

2/17

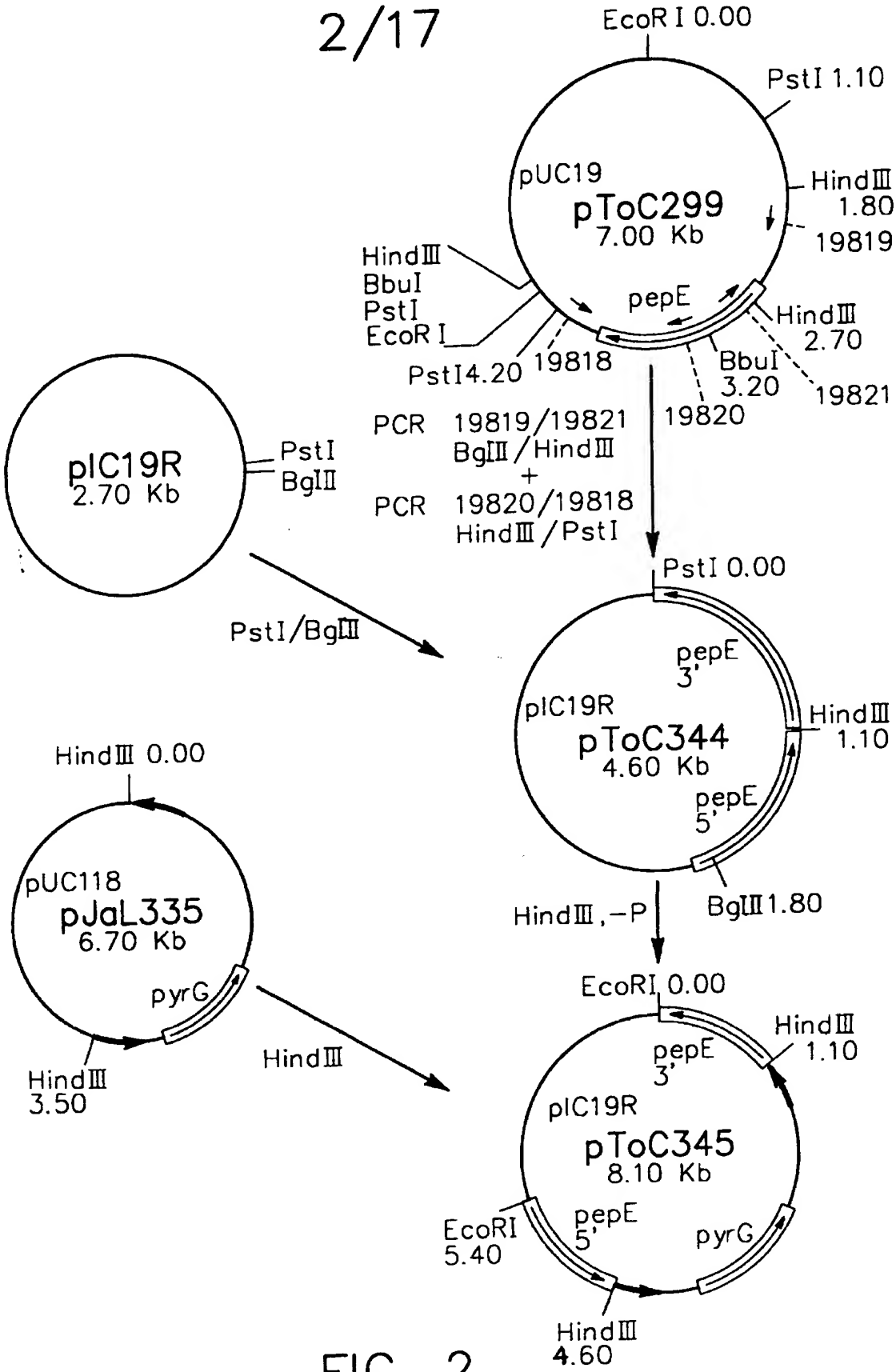

EcoR I 0.00
PstI 1.10
pUC19
pToC299
7.00 Kb
HindIII
1.80
19819
HindIII
BbuI
PstI
EcoR I
pepE
HindIII
2.70
PstI4.20
19818
BbuI
3.20
19821
19820
PCR 19819/19821
BglII/HindIII
+
PCR 19820/19818
HindIII/PstI
pIC19R
2.70 Kb
PstI
BglII
PstI/BglII
PstI 0.00
pepE
3'
pIC19R
pToC344
4.60 Kb
HindIII
1.10
pepE
5'
BglII 1.80
HindIII, -P
HindIII 0.00
pUC118
pJaL335
6.70 Kb
pyrG
HindIII
3.50
HindIII
EcoRI 0.00
HindIII
1.10
pepE
3'
pIC19R
pToC345
8.10 Kb
EcoRI
5.40
pepE
5'
pyrG
HindIII
4.60

FIG. 2

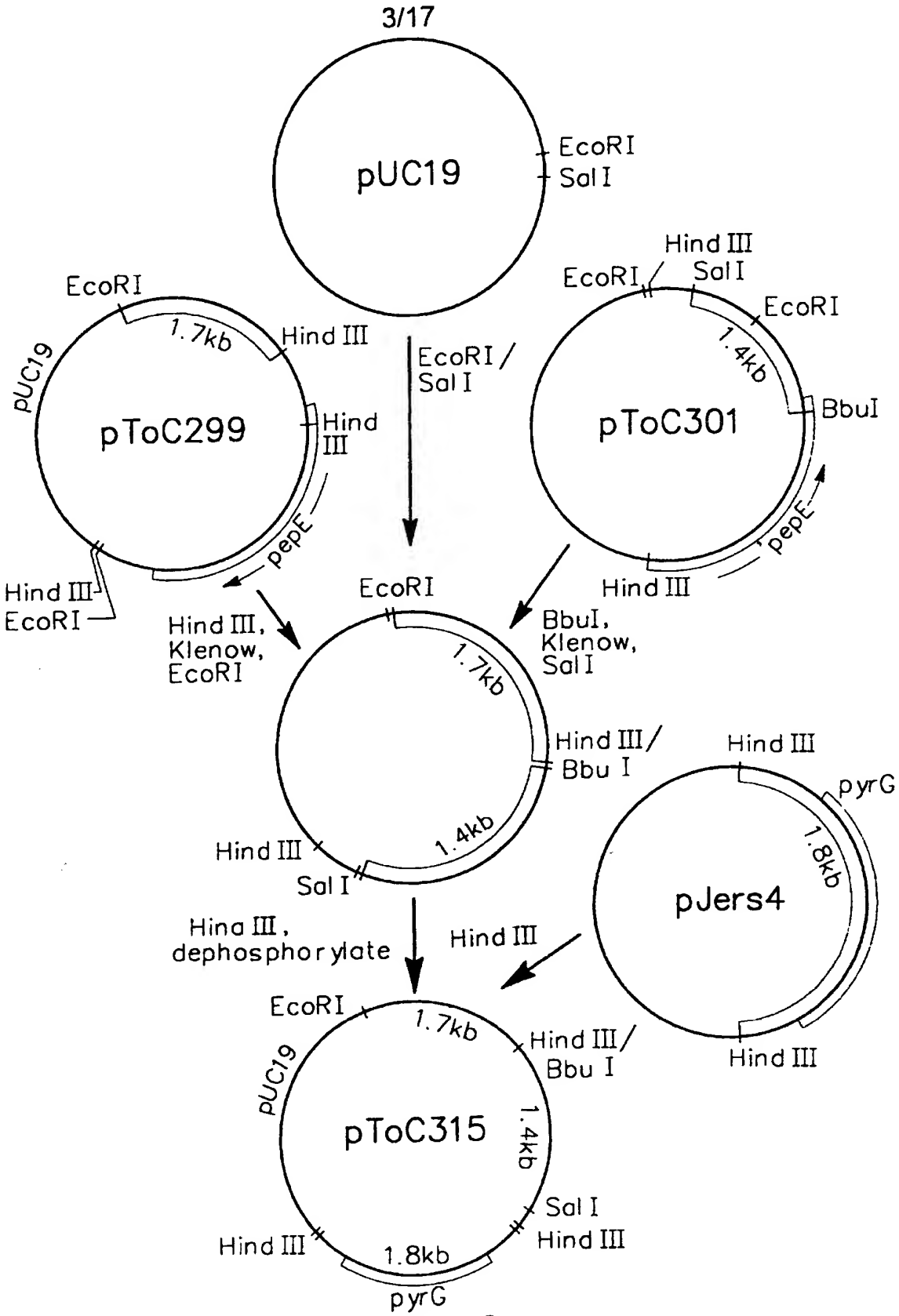

pUC19
EcoRI
Sal I
EcoRI
1.7kb
Hind III
pUC19
pToC299
Hind III
pepE
Hind III
EcoRI
EcoRI /
Sal I
Hind III
Sal I
EcoRI
1.4kb
pToC301
BbuI
pepE
Hind III
Hind III,
Klenow,
EcoRI
EcoRI
1.7kb
Hind III/
Bbu I
Hind III
1.4kb
Sal I
BbuI,
Klenow,
Sal I
Hind III
pyrG
1.8kb
pJers4
Hind III
Hind III,
dephosphorylate
Hind III
EcoRI
1.7kb
Hind III/
Bbu I
pUC19
pToC315
1.4kb
Sal I
Hind III
Hind III
1.8kb
pyrG

FIG. 3

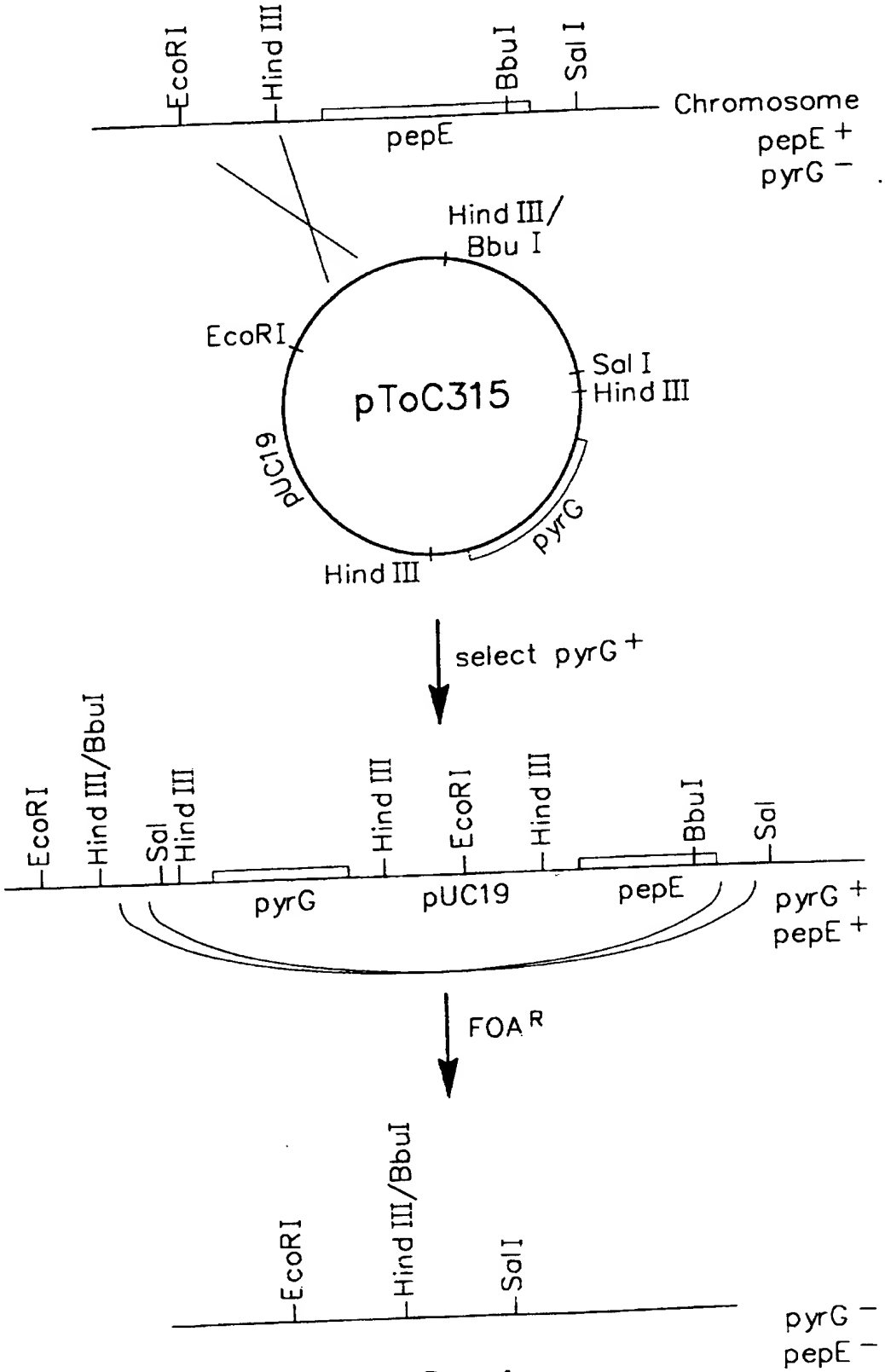

EcoRI
Hind III
BbuI
Sal I
Chromosome
pepE
pepE +
pyrG −
Hind III/
Bbu I
EcoRI
Sal I
Hind III
pToC315
pUC19
pyrG
Hind III
select pyrG +
EcoRI
Hind III/BbuI
Sal
Hind III
Hind III
EcoRI
Hind III
BbuI
Sal
pyrG
pUC19
pepE
pyrG +
pepE +
FOA R
EcoRI
Hind III/BbuI
SalI
pyrG −
pepE −

FIG. 4

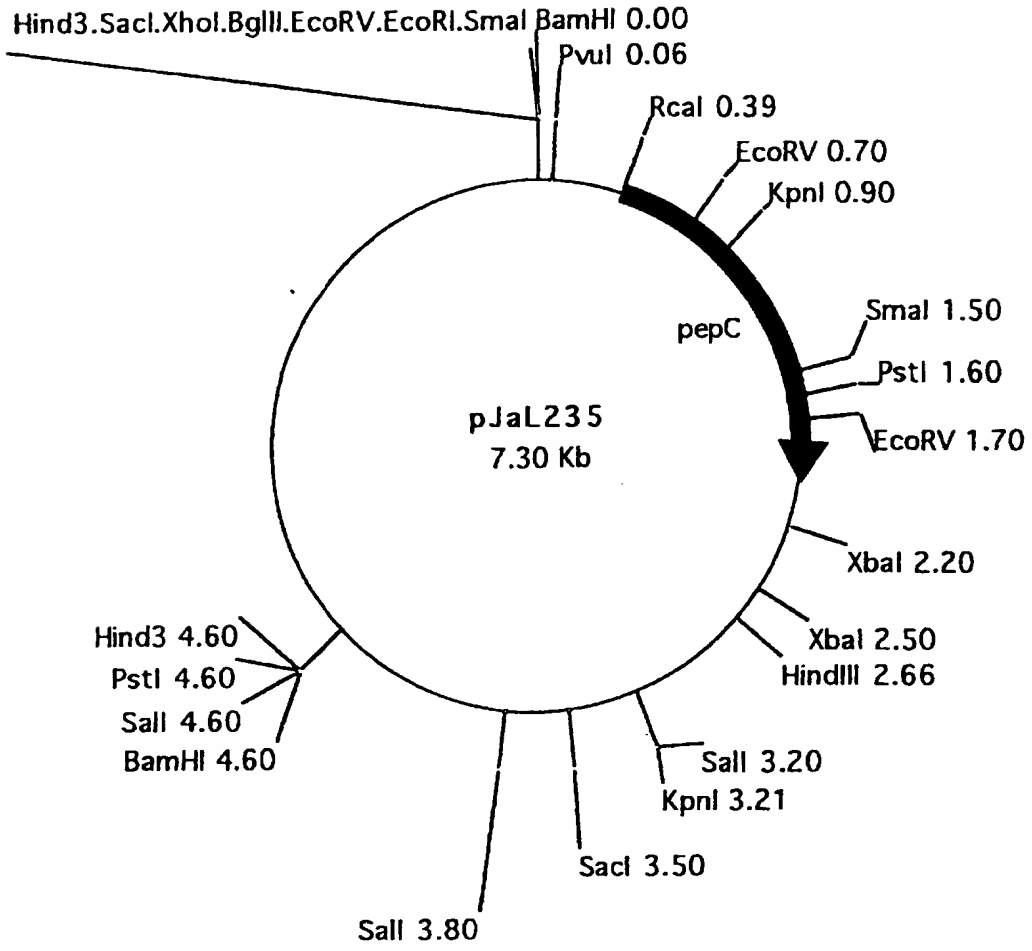

Hind3.SacI.XhoI.BglII.EcoRV.EcoRI.SmaI BamHI 0.00
PvuI 0.06
RcaI 0.39
EcoRV 0.70
KpnI 0.90
SmaI 1.50
PstI 1.60
EcoRV 1.70
XbaI 2.20
XbaI 2.50
HindIII 2.66
SalI 3.20
KpnI 3.21
SacI 3.50
SalI 3.80
Hind3 4.60
PstI 4.60
SalI 4.60
BamHI 4.60
pepC
pJaL235
7.30 Kb

FIG. 5

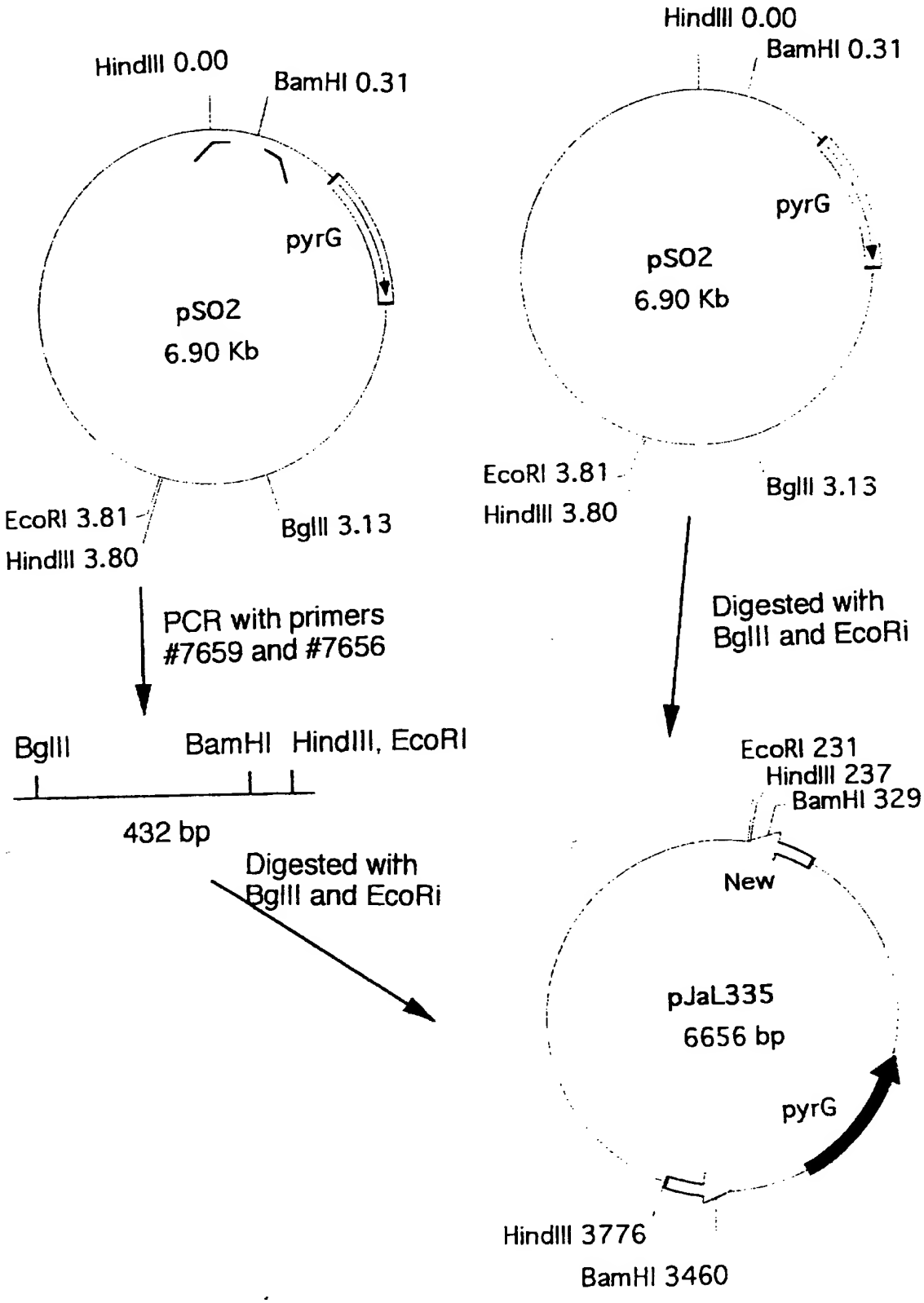

HindIII 0.00
BamHI 0.31
pyrG
pSO2
6.90 Kb
EcoRI 3.81
HindIII 3.80
BglII 3.13
PCR with primers
#7659 and #7656
BglII
BamHI
HindIII, EcoRI
432 bp
Digested with
BglII and EcoRi
HindIII 0.00
BamHI 0.31
pyrG
pSO2
6.90 Kb
EcoRI 3.81
HindIII 3.80
BglII 3.13
Digested with
BglII and EcoRi
EcoRI 231
HindIII 237
BamHI 329
New
pJaL335
6656 bp
pyrG
HindIII 3776
BamHI 3460

FIG. 6

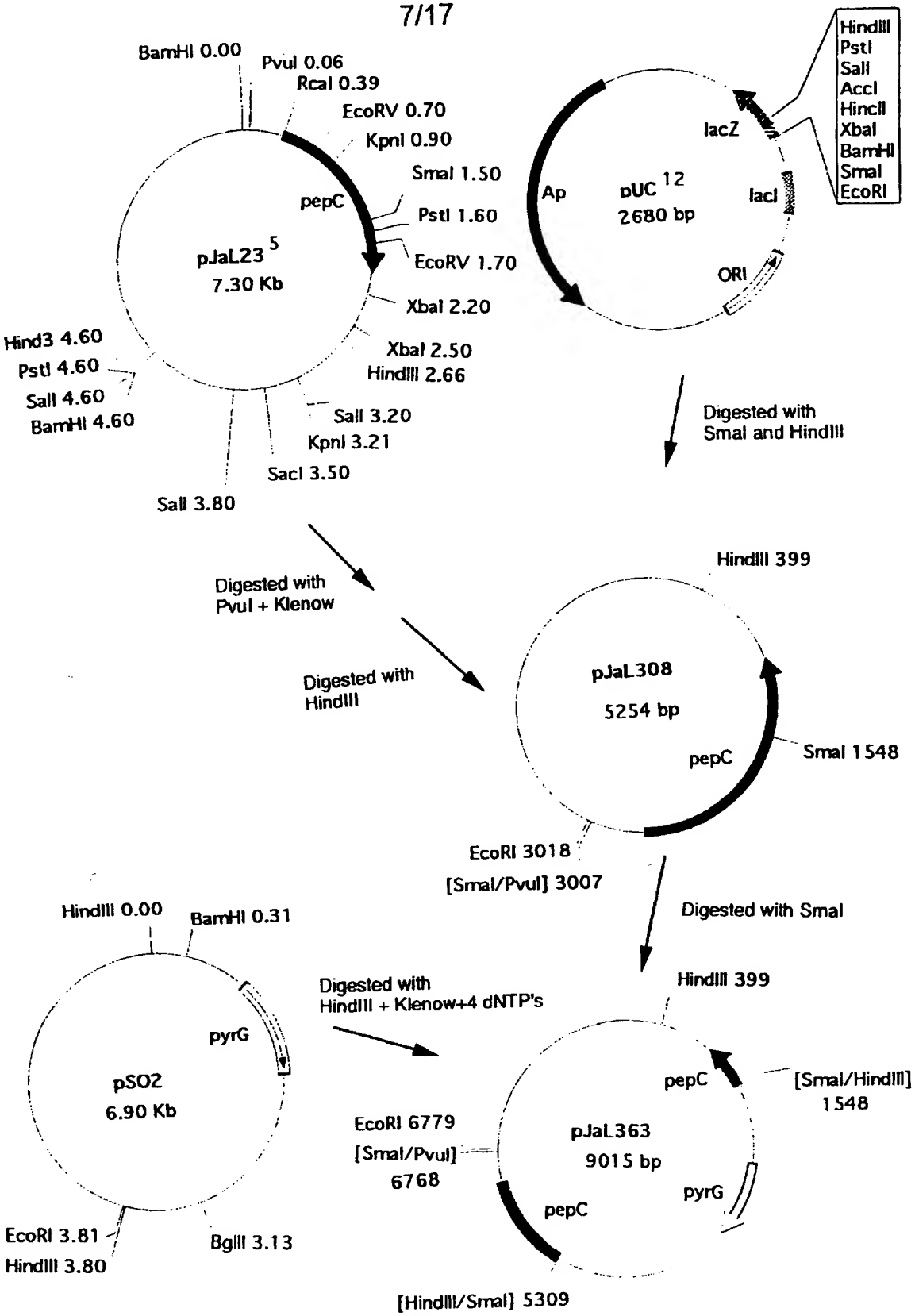

BamHI 0.00
PvuI 0.06
RcaI 0.39
EcoRV 0.70
KpnI 0.90
SmaI 1.50
PstI 1.60
EcoRV 1.70
XbaI 2.20
XbaI 2.50
HindIII 2.66
SalI 3.20
KpnI 3.21
SacI 3.50
SalI 3.80
Hind3 4.60
PstI 4.60
SalI 4.60
BamHI 4.60
pepC
pJaL23 5
7.30 Kb
HindIII
PstI
SalI
AccI
HincII
XbaI
BamHI
SmaI
EcoRI
lacZ
Ap
pUC 12
2680 bp
lacI
ORI
Digested with
SmaI and HindIII
Digested with
PvuI + Klenow
Digested with
HindIII
HindIII 399
pJaL308
5254 bp
pepC
SmaI 1548
EcoRI 3018
[SmaI/PvuI] 3007
Digested with SmaI
HindIII 0.00
BamHI 0.31
pyrG
pSO2
6.90 Kb
EcoRI 3.81
HindIII 3.80
BglII 3.13
Digested with
HindIII + Klenow+4 dNTP's
HindIII 399
pepC
[SmaI/HindIII]
1548
EcoRI 6779
[SmaI/PvuI]
6768
pJaL363
9015 bp
pyrG
pepC
[HindIII/SmaI] 5309

FIG. 7

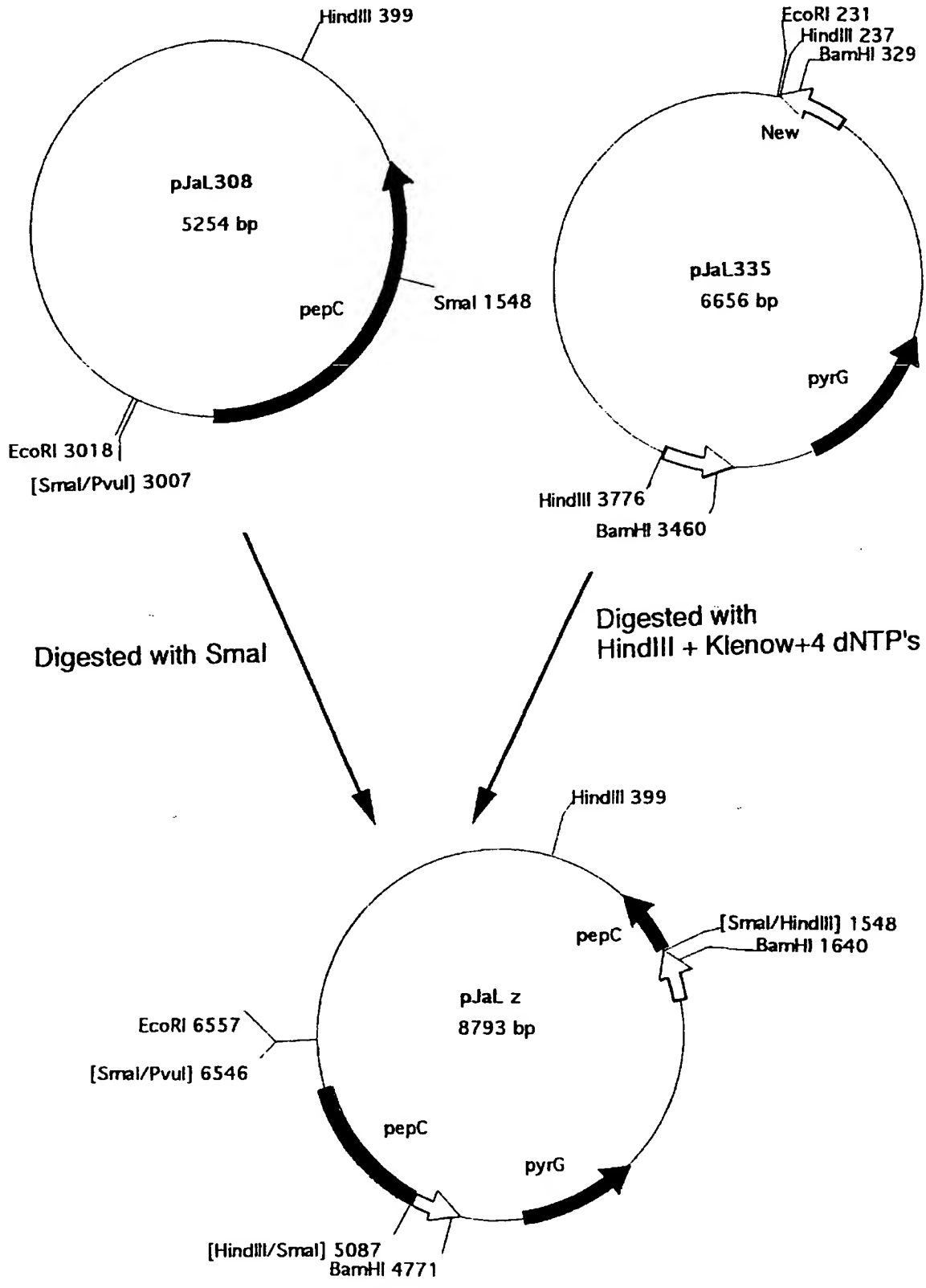
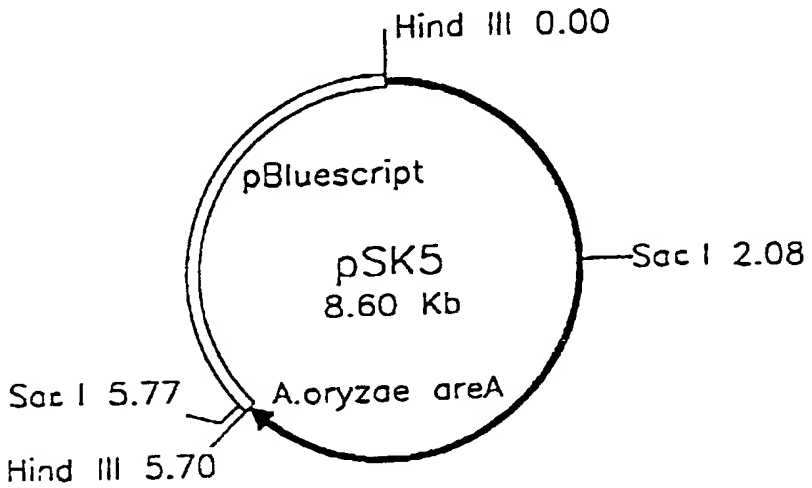

HindIII 399
pJaL308
5254 bp
pepC
SmaI 1548
EcoRI 3018
[SmaI/PvuI] 3007
EcoRI 231
HindIII 237
BamHI 329
New
pJaL335
6656 bp
pyrG
HindIII 3776
BamHI 3460
Digested with SmaI
Digested with
HindIII + Klenow+4 dNTP's
HindIII 399
pepC
[SmaI/HindIII] 1548
BamHI 1640
pJaL z
8793 bp
EcoRI 6557
[SmaI/PvuI] 6546
pepC
pyrG
[HindIII/SmaI] 5087
BamHI 4771

FIG. 8


Hind III 0.00
pBluescript
pSK5
8.60 Kb
Sac I 2.08
Sac I 5.77
A.oryzae areA
Hind III 5.70

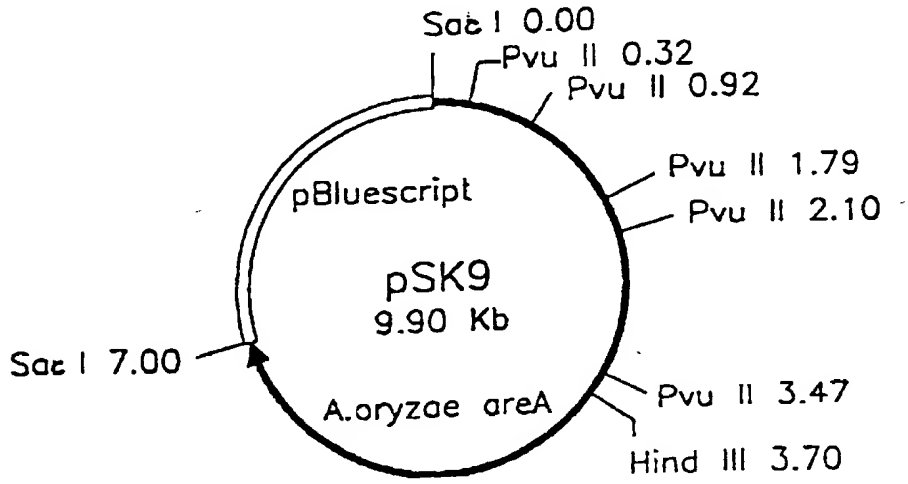

Sac I 0.00
Pvu II 0.32
Pvu II 0.92
Pvu II 1.79
Pvu II 2.10
pBluescript
pSK9
9.90 Kb
Sac I 7.00
A.oryzae areA
Pvu II 3.47
Hind III 3.70

FIG. 9

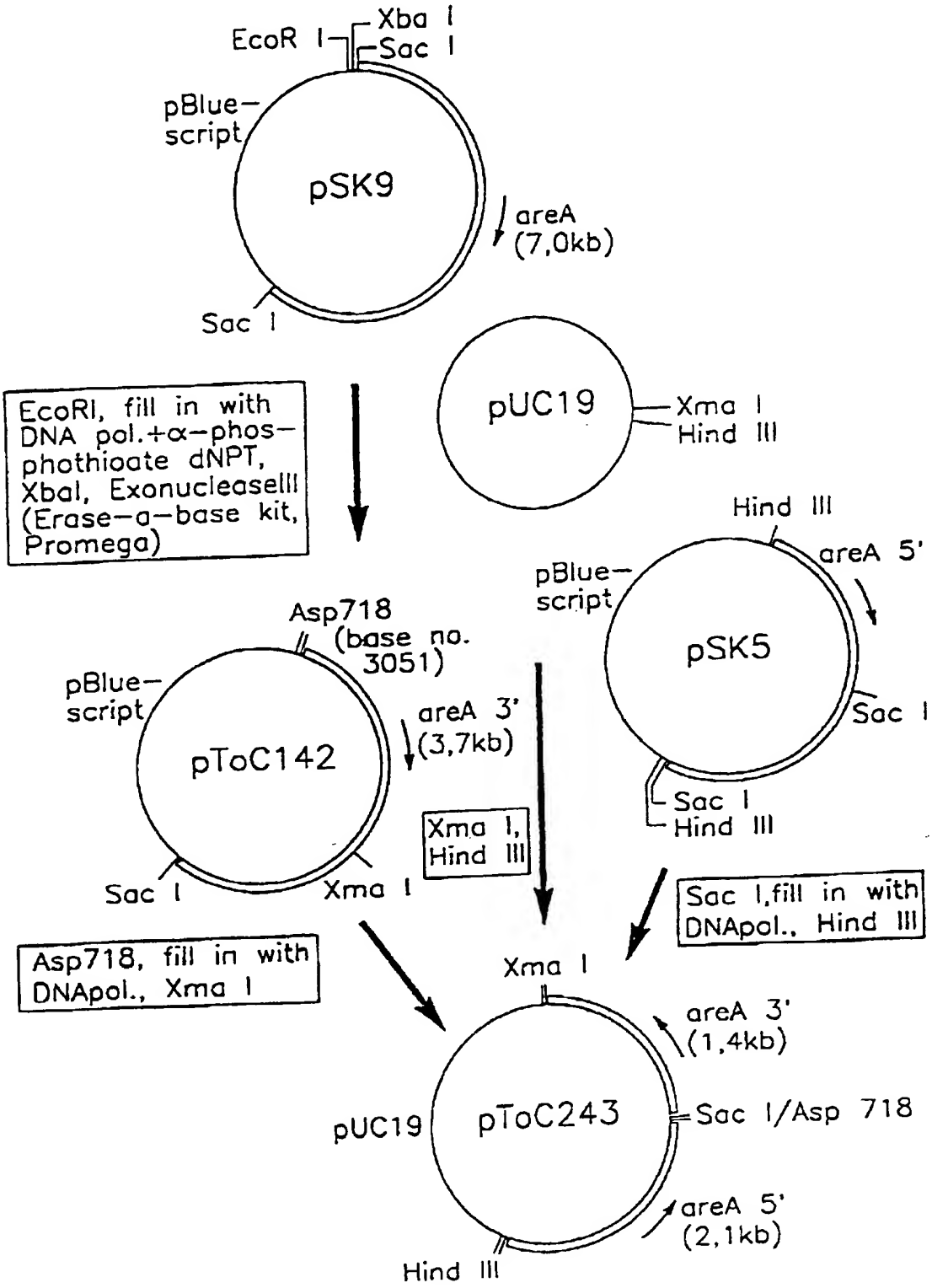

EcoR I
Xba I
Sac I
pBlue-script
pSK9
areA (7,0kb)
Sac I
EcoRI, fill in with DNA pol.+α-phosphothioate dNPT, XbaI, ExonucleaseIII (Erase-a-base kit, Promega)
pUC19
Xma I
Hind III
Hind III
areA 5'
pBlue-script
pSK5
Sac I
Sac I
Hind III
Asp718 (base no. 3051)
pBlue-script
pToC142
areA 3' (3,7kb)
Sac I
Xma I
Xma I, Hind III
Sac I,fill in with DNApol., Hind III
Asp718, fill in with DNApol., Xma I
Xma I
areA 3' (1,4kb)
pUC19
pToC243
Sac I/Asp 718
areA 5' (2,1kb)
Hind III

FIG. 10a

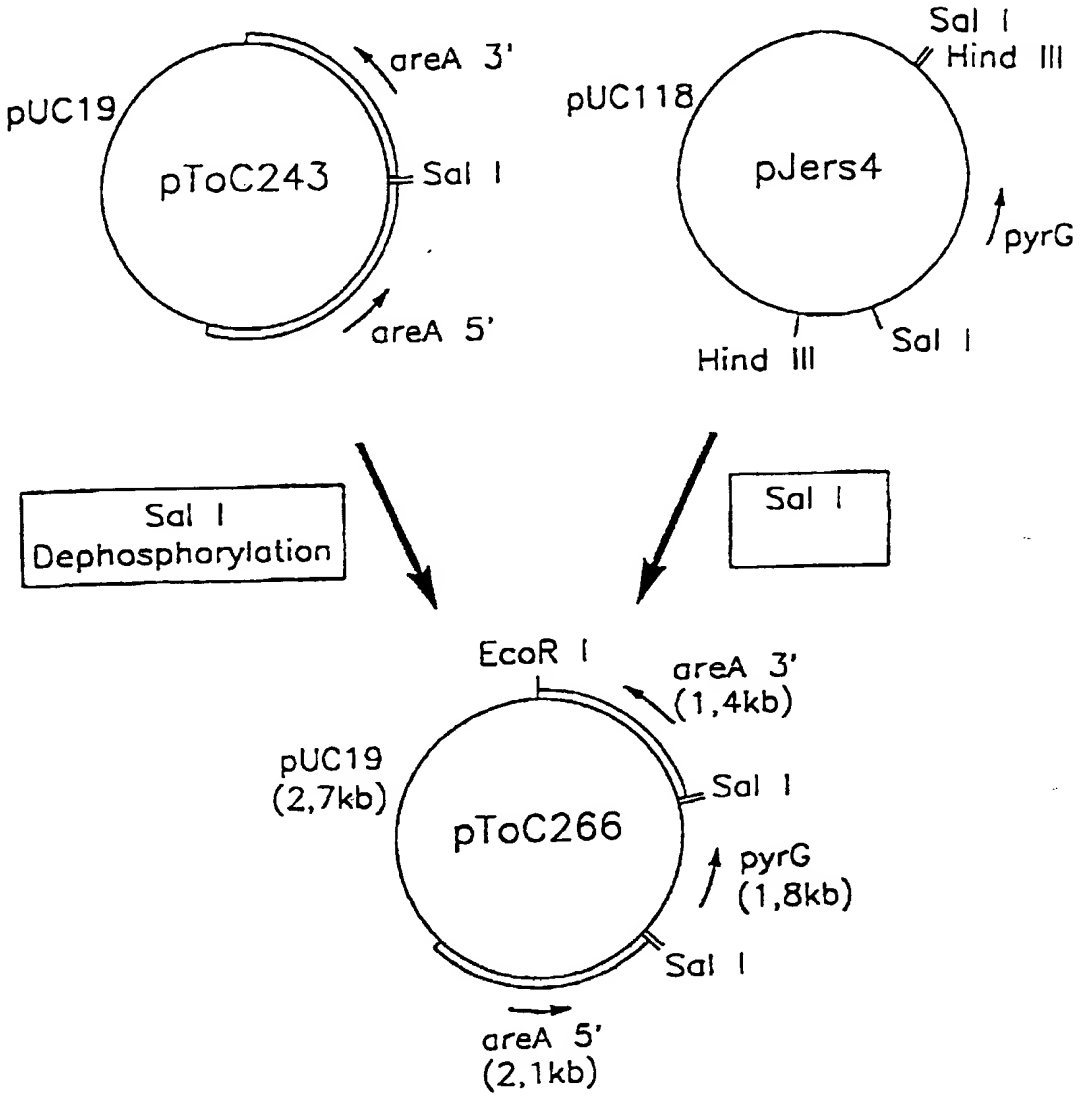

pUC19
pToC243
areA 3'
Sal I
areA 5'
pUC118
pJers4
Sal I
Hind III
pyrG
Hind III
Sal I
Sal I
Dephosphorylation
Sal I
EcoR I
areA 3'
(1,4kb)
pUC19
(2,7kb)
pToC266
Sal I
pyrG
(1,8kb)
Sal I
areA 5'
(2,1kb)

FIG. 10b

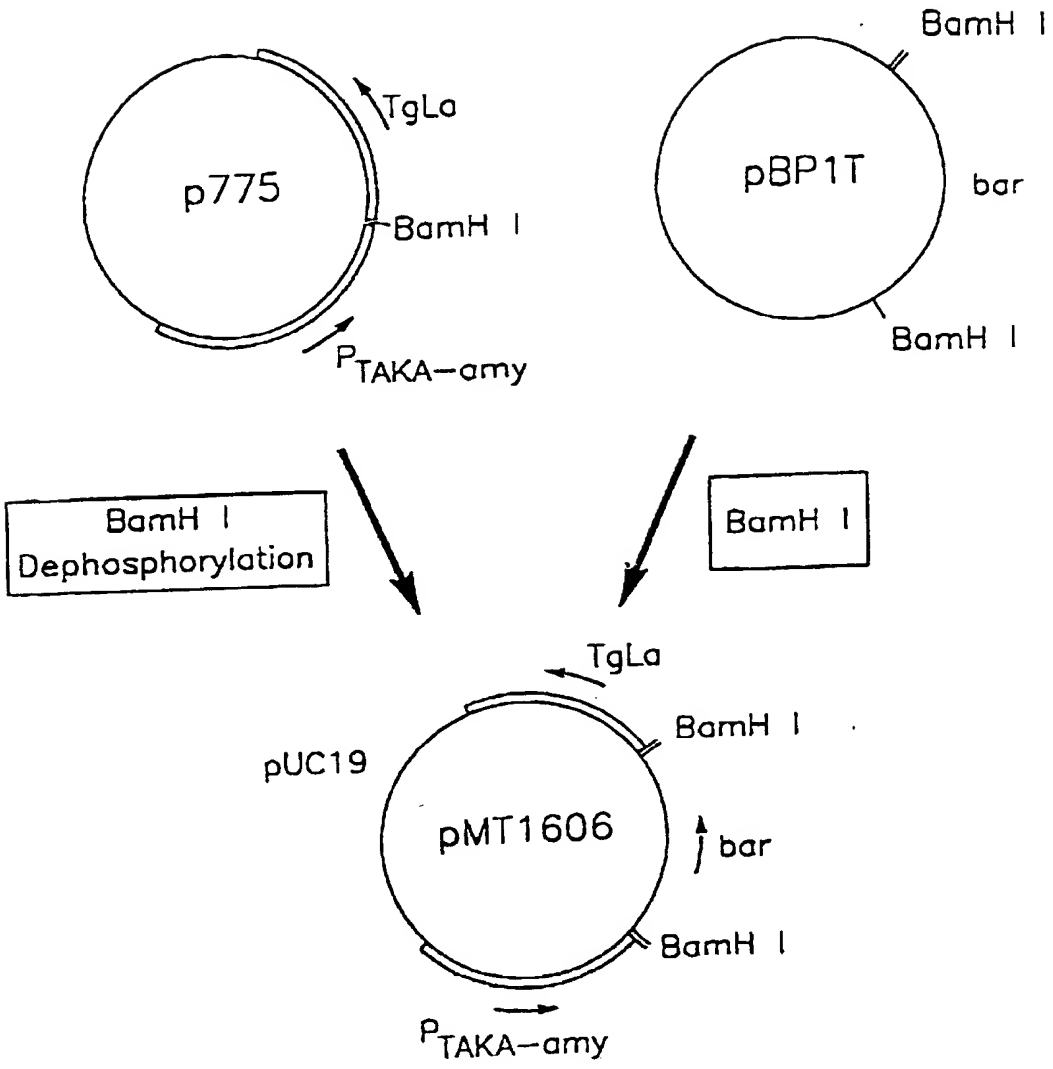

p775
TgLa
BamH I
P TAKA-amy
pBP1T
BamH I
bar
BamH I
BamH I
Dephosphorylation
BamH I
pUC19
pMT1606
TgLa
BamH I
bar
BamH I
P TAKA-amy

FIG. 11

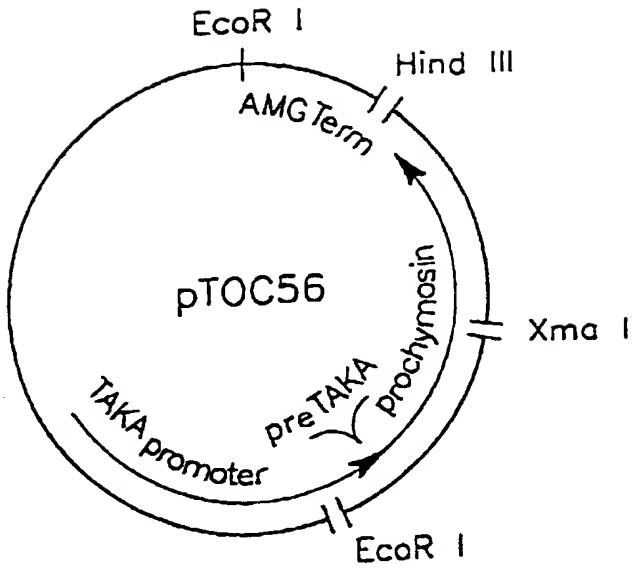

EcoR I
Hind III
AMG Term
pTOC56
prochymosin
Xma I
preTAKA
TAKA promoter
EcoR I

FIG. 12

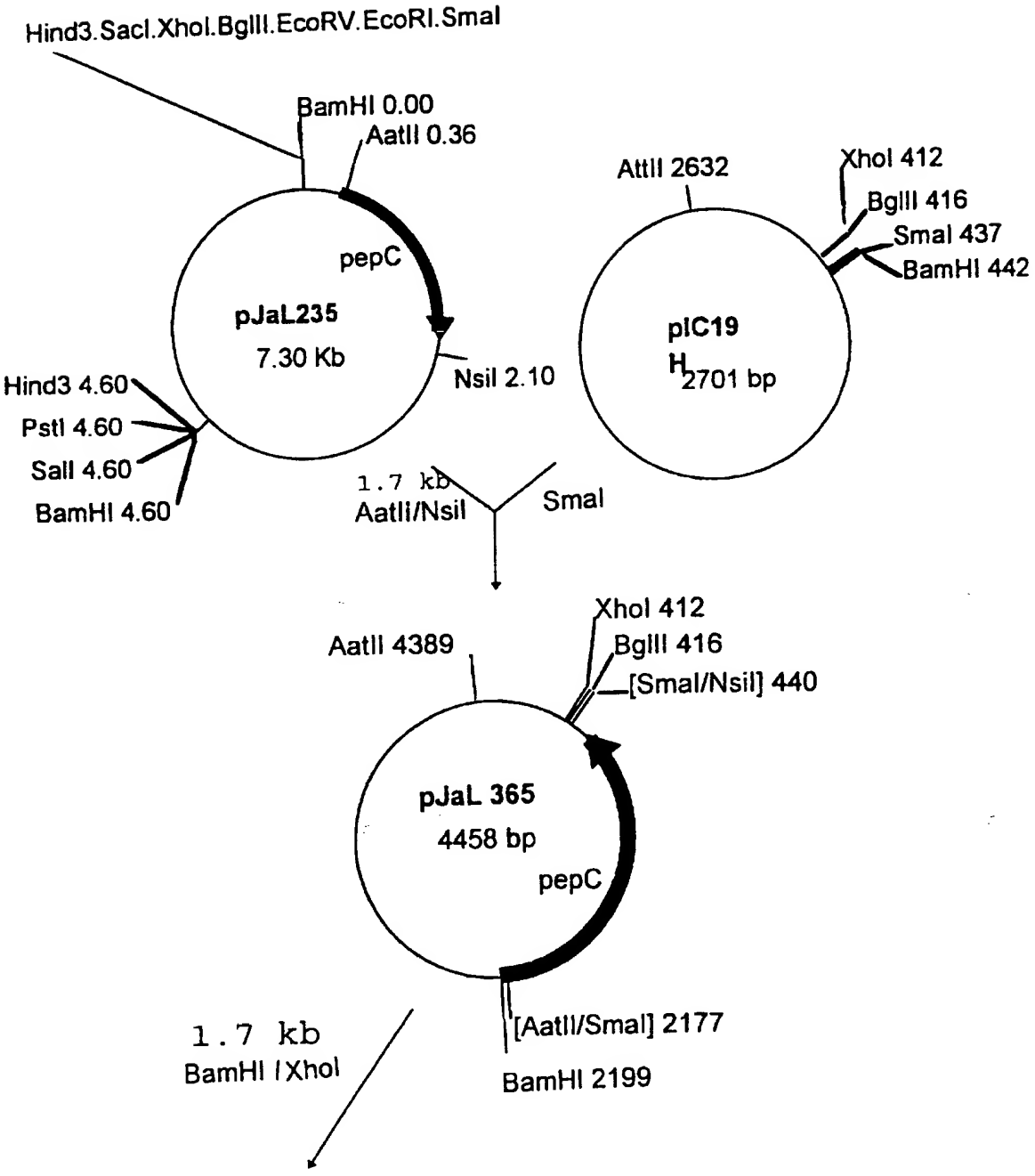

Hind3.SacI.XhoI.BglII.EcoRV.EcoRI.SmaI
BamHI 0.00
AatII 0.36
pepC
pJaL235
7.30 Kb
NsiI 2.10
Hind3 4.60
PstI 4.60
SalI 4.60
BamHI 4.60
AttII 2632
XhoI 412
BglII 416
SmaI 437
BamHI 442
pIC19
H
2701 bp
1.7 kb
AatII/NsiI
SmaI
AatII 4389
XhoI 412
BglII 416
[SmaI/NsiI] 440
pJaL 365
4458 bp
pepC
[AatII/SmaI] 2177
BamHI 2199
1.7 kb
BamHI /XhoI

FIG. 13a

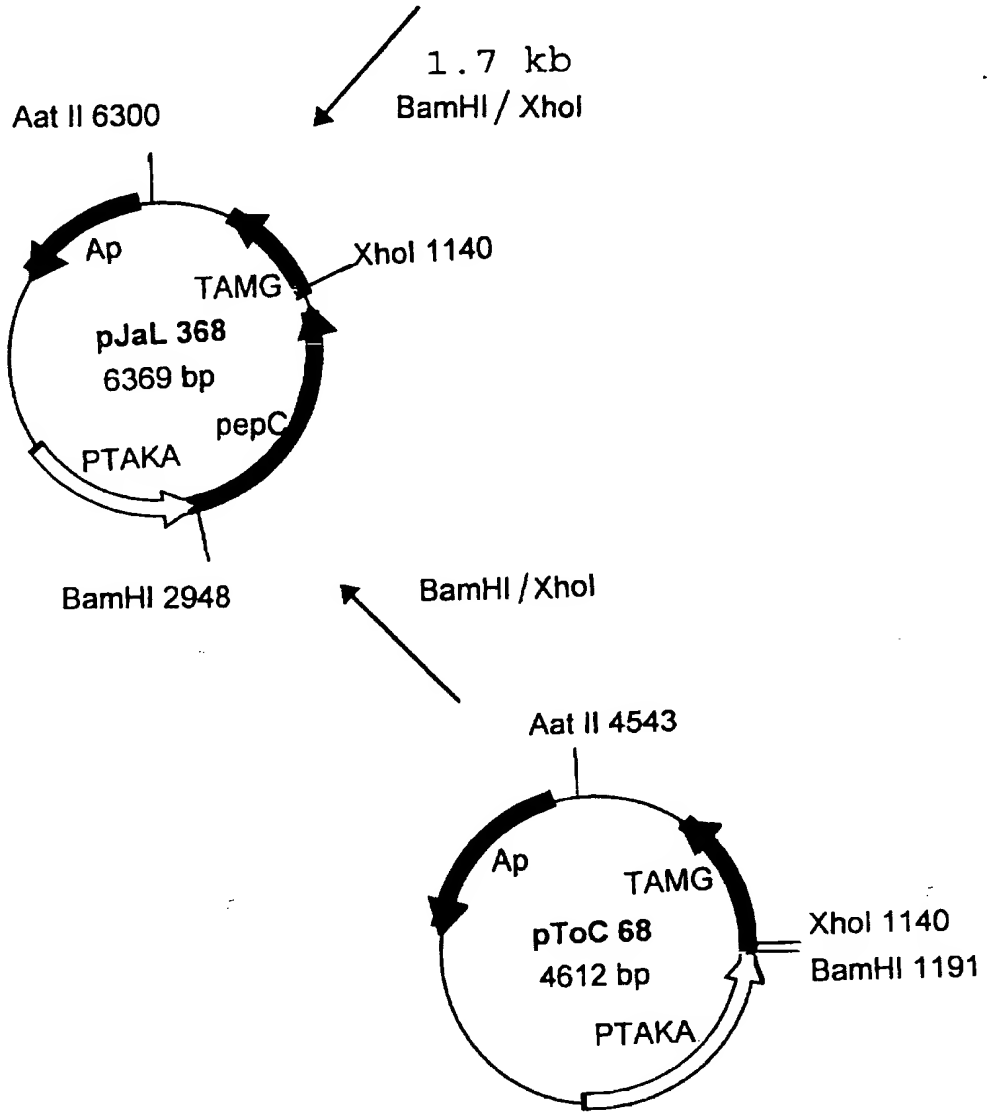

1.7 kb
BamHI / XhoI
Aat II 6300
Ap
TAMG
XhoI 1140
pJaL 368
6369 bp
pepC
PTAKA
BamHI 2948
BamHI / XhoI
Aat II 4543
Ap
TAMG
pToC 68
4612 bp
XhoI 1140
BamHI 1191
PTAKA

FIG. 13b

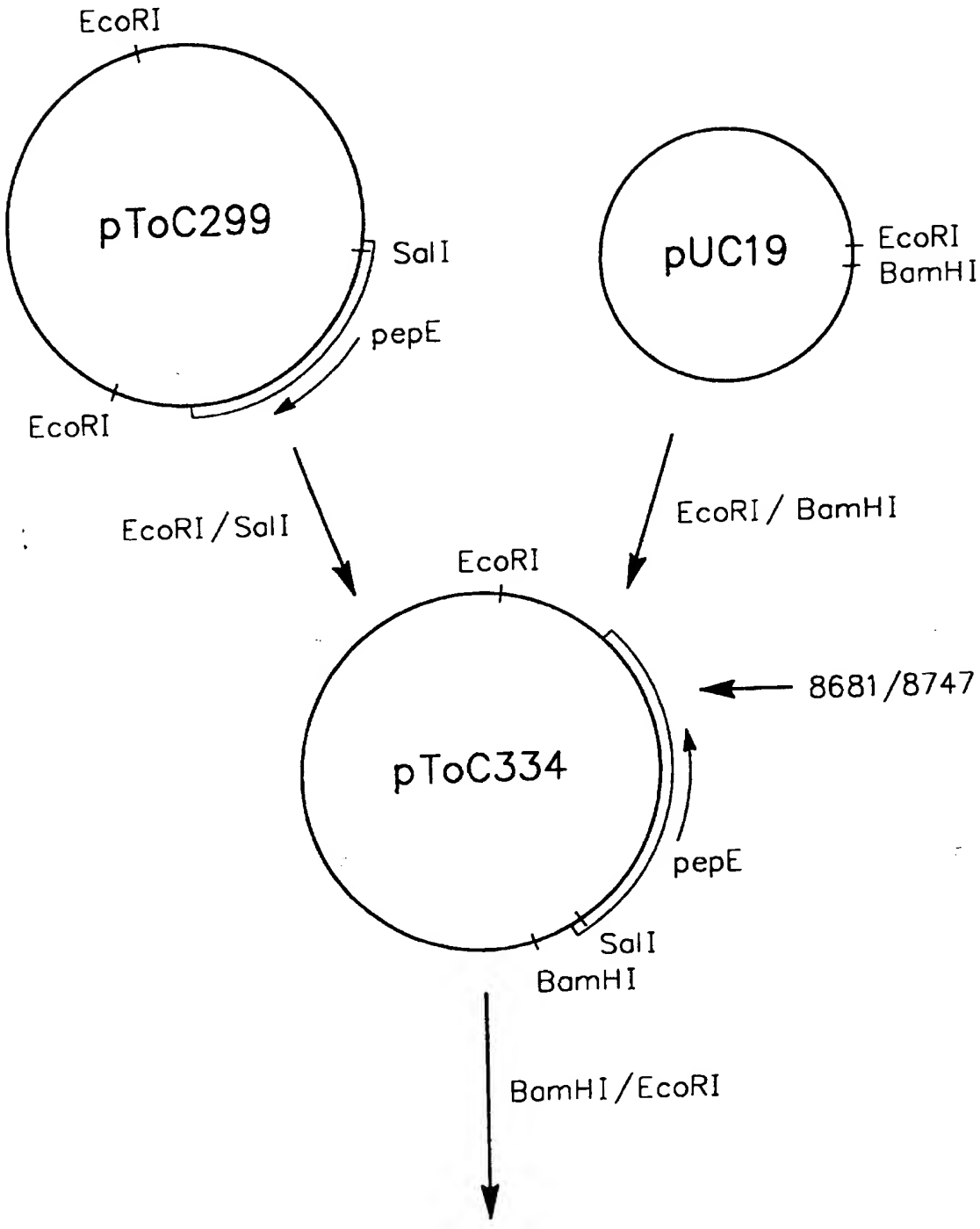

EcoRI
pToC299
SalI
pepE
EcoRI
pUC19
EcoRI
BamHI
EcoRI / SalI
EcoRI / BamHI
EcoRI
8681/8747
pToC334
pepE
SalI
BamHI
BamHI / EcoRI

FIG. 14a

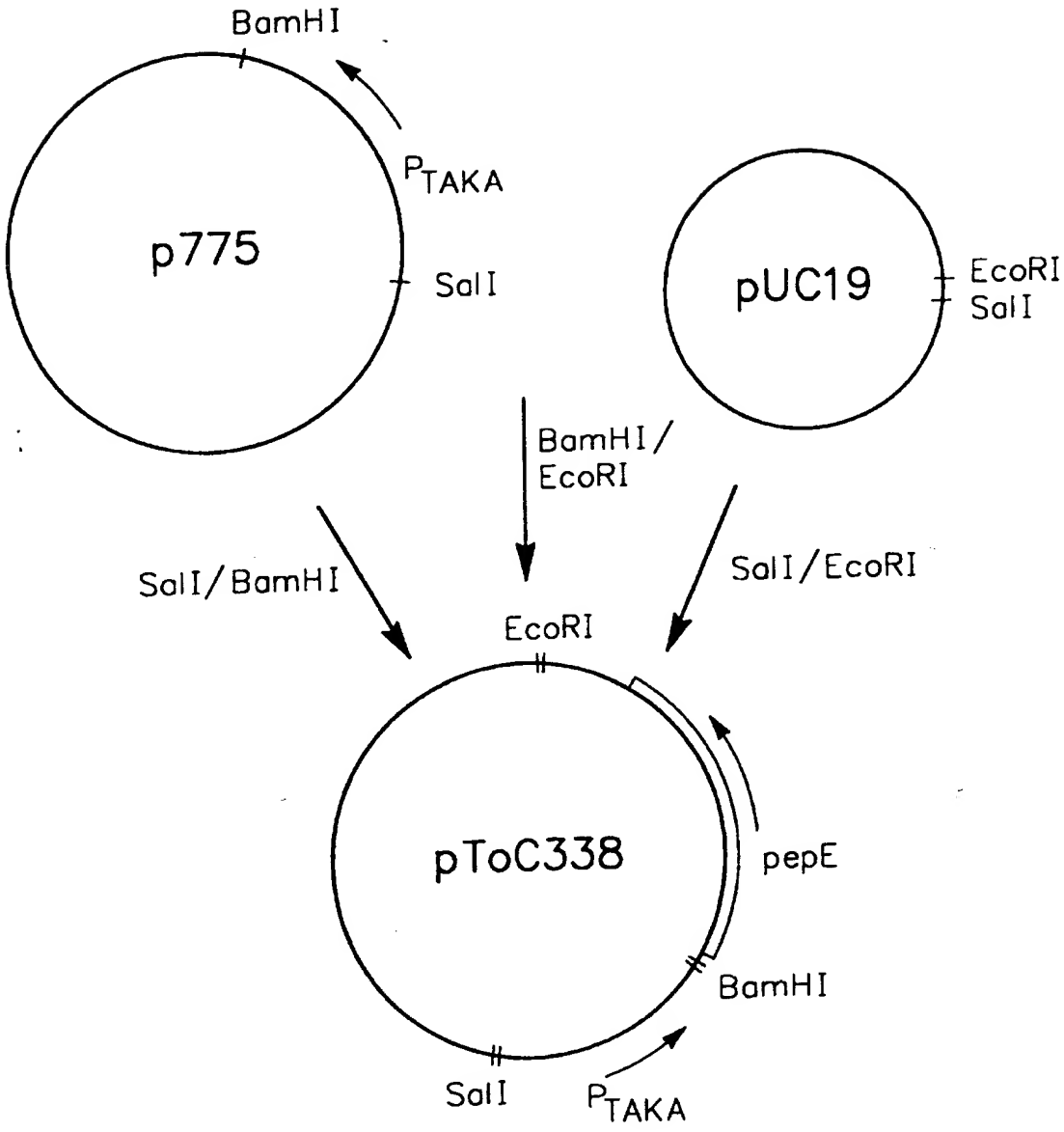

BamHI
P TAKA
p775
SalI
pUC19
EcoRI
SalI
BamHI/
EcoRI
SalI/BamHI
SalI/EcoRI
EcoRI
pToC338
pepE
BamHI
SalI
P TAKA

FIG. 14b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00528

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/80, C12N 1/15
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9535385 A1 (NOVO NORDISK A/S), 28 December 1995 (28.12.95) --	1-27
X	EP 0574347 A2 (CIBA-GEIGY AG), 15 December 1993 (15.12.93), see claims 11-17 --	1-27
A	Molec. gen. Genet., Volume 126, 1973, Herbert N. Arst, Jr. et al, "Nitrogen Metabolite Repression in Aspergillus nidulans" page 111 - page 141 --	1-27

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 1997	15 -05- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00528

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 9217595 A1 (THE SALK INSTITUTE BIOTECHNOLOGY/INDUSTRIAL ASSOCIATES, INC.), 15 October 1992 (15.10.92), see page 4, line 11-28 --	1-27
A	WO 9000192 A1 (GENENCOR, INC.), 11 January 1990 (11.01.90) --	1-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00528

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-27

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Unity of invention is lacking. According to rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same corresponding "special technical features" i.e. features that define a contribution which each of the inventions makes over prior art.

A search for this "special technical feature" mentioned in PCT Rule 13.2 among the independent claims did not reveal such a unifying novel technical feature.

One invention relate to a fungus which does not produce proteases. This is achieved by modifying the areA and PepC and/or PepE genes. Another invention relate to a specific PepE gen and a third invention to a specific PepE gene.

Accordingly the following three inventions were found:

I Claims 1-27 directed to a fungus wherein the areA gene and the PepC and/or PepE genes have been modified

II Claims 28-30 and part of claim 35 directed to a PepC gene from A oryzar (SE Q ID No 1 and 2)

III Claims 31-34 and part of claim 35 directed to a PepE gen from A oryzae (SE Q ID No 2 and 3)

INTERNATIONAL SEARCH REPORT

Information on patent family members

02/04/97

International application No.
PCT/DK 96/00528

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9535385 A1	28/12/95	AU 2733895 A	15/01/96
		FI 965031 A	16/12/96
EP 0574347 A2	15/12/93	AU 663173 B	28/09/95
		AU 3695993 A	21/10/93
		CA 2093950 A	16/10/93
		HU 67800 A	29/05/95
		JP 6046863 A	22/02/94
		NZ 247397 A	28/08/95
		ZA 9302611 A	26/10/93
WO 9217595 A1	15/10/92	AU 661844 B	10/08/95
		AU 1750592 A	02/11/92
		AU 3660095 A	11/01/96
		CA 2105064 A	02/10/92
		EP 0578746 A	19/01/94
		JP 6506117 T	14/07/94
		US 5324660 A	28/06/94
		US 5541112 A	30/07/96
WO 9000192 A1	11/01/90	AT 117720 T	15/02/95
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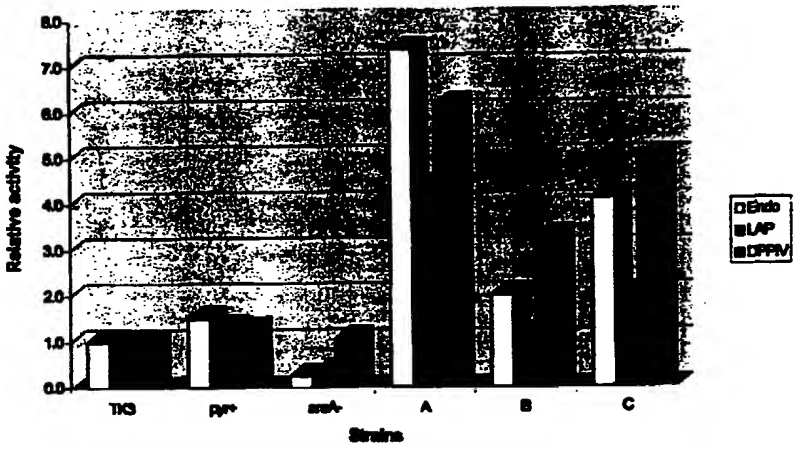

(57) Abstract

The present invention has for object a koji mold which is capable to express at least 2 times more endo– and exo–peptidases than the wild type strain *Aspergillus oryzae* CNCM I–1882, and especially at least 30 mU of endopeptidase activity, at least 30 mU of leucine–amino–peptidase activity and at least 10 mU of prolyldieptidyl–peptidase activity per ml of supernatant when grown in a minimal medium containing 0.2 % soy bean proteins. The invention also provides a DNA–binding protein of *Aspergillus oryzae* (AREA) having at least the amino–acid sequence from amino–acid 1 to amino–acid 731 of SEQ ID NO:2 or functional derivatives thereof. The invention also provides a DNA molecule that comprises an *are*A gene encoding the DNA–binding protein according to the invention. In a fourth aspect, the invention provides a method for over–producing proteolytic enzymes, comprising cultivating a koji mold according to the invention in a suitable growth medium under conditions that the mold expresses enzymes, and optionally isolating the enzymes in the form of a concentrate. In another aspect, the invention provides the use of the koji mold of the invention to hydrolyze protein–containing materials. In a last further aspect, the invention provides a food product comprising a protein hydrolysate obtainable by fermentation with a koji mold of the invention of a material comprising proteins and at least 5mM of L–glutamine.

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Enhanced expression of proteolytic enzymes in koji mold

The invention relates to genetic modifications of koji molds allowing enhanced expression of proteolytic enzymes.

State of the art

Hydrolyzed proteins, which are widely used in the food industry, may be prepared by hydrolysis of protein material with acid, alkali or enzymes. Various methods have been used koji molds for the preparation food products, which are hydrolyzed by action of a large variety of secreted amylases, proteinases and peptidases. Koji molds are those traditionally used for making a koji culture (US4308284) including cells of the genus *Aspergillus*, *Rhizopus* and/or *Mucor*, especially *Aspergillus soyae*, *Aspergillus oryzae*, *Aspergillus phoenicis*, *Aspergillus niger*, *Aspergillus awamori*, *Rhizopus oryzae*, *Rhizopus oligosporus*, *Rhizopus japonicus*, *Rhizopus formosaensis*, *Mucor circinelloides*, *Mucor japanicus*, *Penicillium glaucum* and *Penicillium fuscum*, for example.

According to the rules of the International Code of Botanical Nomenclature (ICBN), *Aspergillus* is an anamorphic genus. This means that true Aspergilli only reproduce asexually through conidiophores. However, the typical *Aspergillus* conidiophore morphology can also be found in fungi that can reproduce sexually via ascospores. Some *Aspergillus* taxonomists caused confusion, because they did not adhere to ICBN terminology. Instead, they attempted to make various revisions of taxonomical schemes to include *Aspergillus nidulans* in this genus, despite the fact that its taxonomically correct name is *Emericella nidulans* (Samson, *In: Aspergillus*. Biology and Industrial Applications, pp 355-390, Ed. by Bennett and Klich, Boston)

EP417481 (Société des Produits Nestlé) thus describes a process for the production of a fermented soya sauce, in which a koji is prepared by mixing a koji culture with a mixture of cooked soya and roasted wheat, the koji is then hydrolyzed in aqueous suspension for 3 to 8 hours at 45°C to 60°C with the enzymes produced during fermentation of the koji culture, a moromi is further prepared by adding sodium chloride to the hydrolyzed koji suspension, the

moromi is left to ferment and is then pressed and the liquor obtained is pasteurized and clarified.

EP429760 (Société des Produits Nestlé) describes a process for the production of a flavoring agent in which an aqueous suspension of a protein-rich material is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH6.0 to 11.0, the suspension is heat-treated at pH 4.6 to 6.5, and the suspension is ripened with enzymes of a koji culture.

Likewise, EP96201923.8 (Société des Produits Nestlé) describes a process for the production of a meat flavor, in which a mixture containing a vegetal proteinaceous source and a vegetal carbohydrates containing source is prepared, said mixture having initially at least 45% dry matter, the mixture is inoculated with a koji culture and by one or more another species of microorganisms involved in the traditional fermentation of meat, and the mixture is incubated until meat flavors are formed.

However, on the one hand, acid or alkaline hydrolysis can destroy the essential amino acids produced during hydrolysis thus reducing the nutritional value, whereas enzymatic hydrolysis rarely goes to completion so that the hydrolyzed protein contains substantial amounts of peptides. The optimization and further development of koji processes have been seriously hampered by the lack of knowledge on the nature of the hydrolytic enzymes, their regulation and how process parameters affect their expression and activity (e.g. temperature, pH, water activity, and salt concentration).

In the fungal *Emericella nidulans* (Katz *et al.*, Gene, 150, 287-292, 1994), fermentation activity is subject to at least three general control circuits including carbon catabolite repression, nitrogen and sulfur metabolite repression. These three regulatory circuits ensure that the available nitrogen-, carbon-, and sulfur sources in a substrate are utilized sequentially according to their nitrogen, energy and sulfur yield. Nitrogen metabolite repression is exerted by the *are*A gene product in *Emericella nidulans* (Arst *et al.*, Mol. Gen. Genet., 26, 111-141, 1973), whereas in the other fungals *Neurospora crassa* (Davies *et al.*, Proc. Natl. Acad. Sci. USA, 84, 3753-3757, 1987), *Penicillium chrysogenum* (Haas *et al.*, Curr.

Genet., 27, 150-158, 1995) and *Saccharomyces cerevisiae* (Minehart *et al.*, Mol. Cell. Biol., 11, 6216-6228, 1991) similar genes exert a similar function.

The *are*A gene encodes a positively acting DNA-binding protein (AREA), belonging to the GATA family of transcription factors, that is required for the utilization of all nitrogen sources except ammonia or L-glutamine. Under nitrogen de-repressed conditions, signaled by high intracellular levels of glutamine, *are*A expression is down regulated by three mechanisms: 1) the AREA protein is inactivated, 2) *are*A transcription is halted and 3) by action of the 3' untranslated trailer sequence (3'-UTS) *are*A mRNA degradation is enhanced (Platt *et al.*, EMBO J., 15, 2791-2801, 1996). In the absence of a functional AREA protein, only ammonia or L-glutamine can be utilized as nitrogen source. Consequently, loss-of-function *are*A mutants can utilize only ammonia or L-glutamine as nitrogen sources (Arst *et al.*, 1973).

Observations in koji fermentation suggest that nitrogen metabolite repression is a major parameter in koji fermentation. For instance, high levels of L-glutamine are shown to negatively affect proteolytic activity in koji fermentation.

Furthermore, it has been observed that high levels of proteolytic activity and glutaminase activity are two mutually exclusive conditions in koji fermentation (Ushijima *et al.*, Agric. Biol. Chem., 51, 1051-1057, 1997). For instance, addition of 25mM L-glutamine into a minimal growth medium containing 0.1% wheat gluten reduces endoproteolytic enzyme activity about 40-50 fold. This phenomenon may be explained by postulating that L-glutamine is necessary for the induction of glutaminase. However, since L-glutamine is also the effector of nitrogen metabolite repression, the expression of proteolytic enzymes is suppressed when glutaminase is induced.

With regard to the fact that glutaminase suitably converts L-glutamine into L-glutamic acid which is an important natural taste enhancer (see WO95/31114), there is hence a need to overcome L-glutamine mediated suppression of proteolytic enzymes, allowing simultaneous expression of glutaminase and proteolytic enzymes in koji molds.

In addition, depending on the nature of the protein and the enzymes used for proteolysis, the peptides formed can however have extremely bitter tastes and are thus organoleptically undesirable. There is hence also a need for methods of hydrolyzing proteins leading to high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties.

Finally, biochemical analysis of residual peptides in cereals hydrolyzed by *koji* molds, e.g. wheat gluten, shows that a considerable amount of L-glutamine remains sequestered in proline containing peptides (Adler-Nissen, *In:* Enzymatic hydrolysis of food proteins. Elsevier Applied Sciences Publishers LTD, p120, 1986). There is hence also a need for methods of hydrolyzing proteins leading to liberation of high amount of L-glutamine.

Summary of the invention

The present invention has for object a koji mold which is capable to express at least 2 times more endo- and exo-peptidases than the wild type strain *Aspergillus oryzae* CNCM I-1882, and especially at least 30 mU of endopeptidase activity, at least 30 mU of leucine-amino-peptidase activity and at least 10 mU of prolyl-dipeptidyl-peptidase activity per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins.

In a second aspect, the invention also provides a DNA-binding protein of *Aspergillus oryzae* (AREA) having at least the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2 or functional derivatives thereof.

In a third aspect, the invention provides a DNA molecule that comprises an *are*A gene encoding the DNA-binding protein according to the invention.

In a fourth aspect, the invention provides a method for over-producing proteolytic enzymes, comprising cultivating a koji mold according to the invention in a suitable growth medium under conditions that the mold expresses enzymes, and optionally isolating the enzymes in the form of a concentrate.

In another aspect, the invention provides the use of the koji mold of the invention to hydrolyze protein-containing materials.

In a last further aspect, the invention provides a food product comprising a protein hydrolysate obtainable by fermentation with a koji mold of the invention of a material comprising proteins and at least 5mM of L-glutamine.

Detailed description of the invention

Within the following description, the percentages are given by weight except where otherwise stated. The amino acid or nucleotide sequences referred as "SEQ ID NO:" are always presented in the sequence listing hereafter. One leucine-aminopeptidase enzyme unit is defined as the amount of enzyme which produces 1 µmol *p*-nitroaniline per minute at 37°C from the substrate leucine-*p*-nitroanilide (absorption measured at 400nm; ε= 10'500 $M^{-1}cm^{-1}$). One prolyl-dipeptidyl-peptidase enzyme unit is defined as the amount of enzyme which produces 1 µmol *p*-nitroaniline per minute at 37°C from the substrate Alanine-Proline-*p*-nitroanilide (absorption measured at 400nm; ε= 10'500 $M^{-1}cm^{-1}$). One endopeptidase enzyme unit is defined as the amount of enzymes which produces 1 µmol of TCA-soluble peptides per minute at 37°C from the resorufin-labeled casein substrate under prescribed conditions (Boehringer Cat No. 1080733; absorption measured at 574nm).

The term "koji" is defined as the product of the fermentation with a koji mold culture of a mixture of a source of proteins and a source of carbohydrates, especially of a mixture of a leguminous plant or of a cooked oleaginous plant and of a cooked or roasted cereal source, for example of a mixture of soya or cooked beans and of cooked or roasted wheat or rice.

Likewise, the expression "functional derivative of an enzyme" includes all amino acid sequences which differ by substitution, deletion, addition of some amino acids, for instance 1-20 amino acids, but which keep their original activities or functions. The selection of a functional derivative is considered to be obvious to one skilled in the art, since one may easily creates variants of the truncated AREA protein (see SEQ ID NO:2) by slightly adapting methods known to one skilled in the art, for instance the methods described by Adams *et al.* (EP402450; Genencor), by Dunn *et al.* (Protein Engineering, 2, 283-291, 1988), by Greener *et al.* (Strategies, 7, 32-34, 1994), and/or by Deng *et al.* (Anal. Biochem, 200, 81, 1992).

In particular, a protein may be generally considered as a derivative to another protein, if its sequence is at least 85% identical to the protein, preferably at least 90%, in particular 99%. In the context of the present disclosure, the identity is determined by the ratio between the number of amino acids of a derivative sequence which are identical to those of the truncated AREA protein (see SEQ ID NO:2) and the total number of or amino acids of the said derivative sequence.

The present invention thus concerns any koji molds providing an enhanced expression of proteolytic enzymes, leading to high degree of protein hydrolysis and to hydrolysates with excellent organoleptic properties. Accordingly, these koji molds express (1) high levels of endopeptidases such as those capable to produce TCA-soluble peptides at 37°C from casein, and (2) high levels of exo-peptidases such as the leucine-amino-peptidase that eliminates N-terminal leucines (Deng *et al.*, Anal. Biochem., 200, 81, 1992) and the prolyl-dipeptidyl-peptidase which eliminates N-terminal X-Proline dipeptides, wherein X may be any amino-acid (Barrett *et al.*, In Mammalian Proteases: A Glossary and Bibliography, N.Y., Acad. Press, 2, p.132, 1986).

With regard to the fact that koji molds of the invention provide an enhanced prolyl-dipeptidyl-peptidase activity, they may suitably be used for liberating L-glutamine remains sequestered in proline containing peptides.

Koji molds providing the following enhanced expression of proteolytic enzymes are particularly adapted for the purpose of the invention: at least about 30 mU/ml*, preferably at least about 50 mU/ml* of endopeptidase activity; at least about 30 mU/ml*, preferably at least about 50 mU/ml* of leucine-amino-peptidase activity; and at least 10 mU/ml*, preferably at least about 15 mU/ml* of proline-dipeptidyl-peptidase activity (* per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins).

In addition, koji molds that overcome L-glutamine mediated suppression of proteolytic enzymes, allowing simultaneous expression of glutaminase and proteolytic enzymes, are also part of the invention. These koji molds thus may express the above-mentioned proteolytic activities when grown in a minimal

medium containing 0.2% soy bean proteins and at least 5 mM L-glutamine (0.073% w/w), for instance.

Koji molds of the invention may be obtained by random U.V and/or chemical mutagenesis, followed by selection of mutagenised koji mold providing the required phenotypic characteristics.

Selection of mutagenised koji mold particularly containing a mutagenised *are*A gene which is not repressed, when the mutagenised mold is grown in a minimal medium containing repressive amounts of L-glutamine, suitably achieved the needs of the present invention. To this end, *are*A mutants may be easily selected by classical random mutagenesis (UV, chemical) and selection on plates containing about 100 mM methyl ammonium chloride and 0.2% soy protein, for example.

It has to be noted that the prolyl-dipeptidyl-peptidase activity that is not naturally controlled by the *are*A gene expression, is enhanced against all expectations when the *are*A gene is de-repressed. Since expression of the prolyl-dipeptidyl-peptidase activity is induced by peptides (unpublished results), this AREA-dependent increase in activity may in fact be caused by the enhanced liberation of peptides by the endoproteases that are under *are*A control.

With regard to the fact that random U.V and/or chemical mutagenesis is time consuming, it would be also more adequate to construct koji molds of the invention by recombinant technology. Accordingly, a koji mold of the invention may preferably contain a recombinant *are*A gene which is truncated so as the C-terminally truncated AREA protein remains functional but not repressed when the mold is grown in a minimal medium containing repressive amounts of L-glutamine. It has to be noted that this truncation leads also to an *are*A mRNA that is less sensitive to mRNA degradation.

Truncation may be effected by cutting the native *are*A gene to a pre-determined region, and by introducing a terminater region thus allowing transcription of a truncated *areA* mRNA. Truncation is preferably effected downstream of the sequence encoding the DNA binding domain of AREA, that can be easily identified by 17 amino acid loop bound two pairs of cystein residues. More

precisely, truncation may be effected downstream of the *are*A sequence encoding the conservative amino-acid structure cystein-2X-cystein-17X-cystein-2X-cystein, wherein X is any amino-acids and the numbers 2 and 17 refer to the number of amino-acids (Caddick *et al.*, Antonie van leeuwenhoek, 65, 169-177, 1994). This truncation may be particularly carried out in the 100 amino-acids following the *are*A sequence encoding the DNA binding domain.

Any functional fungal *are*A gene may be used in the context of the present invention, and in particular any functional *are*A gene capable of hybridizing under stringent conditions to the *are*A gene of *Aspergillus oryzae* having the nucleotide sequence from nucleotide 1189 to nucleotide 3846 of SEQ ID NO:1 or functional derivatives thereof due to the degeneracy of the genetic code.

A functional *are*A gene may be obtained in substantially purified form by using the method described within the following examples from any strain of *Aspergillus oryzae*. Alternatively, an *are*A gene may be (1) detected also from other genera or species of fungals by use of DNA probes derived from the nucleotide sequence SEQ ID NO:1 in a stringent hybridization assay, and (2) recovered by the well known Reverse-PCR method by use of suitable primers derived from SEQ ID NO:1 encompassing the *areA* gene. In a further aspect, an *are*A gene may also be *in-vitro* synthesized and then multiplied by using the polymerase chain reaction, for instance.

A suitable truncated *are*A gene thus may particularly consist of the nucleotide sequence defined by nucleotides 1189-1604 and 1704-3480 of SEQ ID NO:1 (SEQ ID NO: 1 contains an intron) or functional derivatives thereof due to the degeneracy of the genetic code, for example. This truncated gene thus encodes for the AREA DNA-binding protein of *Aspergillus oryzae* having the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2, that is required for the utilization of all nitrogen sources except ammonia or L-glutamine.

This truncated *are*A gene then may be introduced in a vector, e.g. a replicative plasmid or an integrative circular or linearized non replicative plasmid, and may be operably linked to regulatory sequences that regulate a different gene in the said organism of origin or that regulate a different gene in a foreign organism (promoter and/or a terminator), for example. A regulatory sequence other than the

native regulatory sequence will generally be selected for its high efficiency or desirable characteristic, such as, in case of a promoter inducibility or high expression capacity, for example.

If heterologous expression is preferred, meaning that the gene of the invention is expressed in another organism than the original host (strain, variety, species, genus, family, order, class or division) the regulatory sequences are preferably derived from an organism similar or equal to the expression host. For example, if the expression host is an *Aspergillus*, then the regulatory sequences will be derived from *Aspergillus*. The promoter suitable for constitutive expression, preferably in a fungal host, may be a promoter from the following genes: glycerolaldhehyde-3-phosphate dehydrogenase, phospho-glycerate kinase, triose phosphate isomerase and acetamidase, for example. Promoter suitable for inducible expression, preferably in a fungal host, may be a promoter from the following genes: endoxylanase IIA, glucoamylase A, cellobiosehydrolase, amylase, invertase, alcohol dehydrogenase and amyloglucosidase. The selection of a desirable regulatory sequence operably linked to a sequence of the invention and capable of directing the expression of the said nucleotide sequence is considered to be obvious to one skilled in the art.

The vector may also comprise a selection marker to discriminate host cells into which the recombinant DNA material has been introduced from cells that do not comprise the said recombinant material. Such marker genes are, for example in case fungal expression is preferred, the known *ga*-2, *pyr*G, *pyr*4, *pyr*A, *trp*C, *amd*S or *arg*B genes. The DNA molecule may also comprise at least one suitable replication origin. Suitable transformation methods and suitable expression vectors provided with a suitable transcription promoter, suitable transcription termination signals and suitable marker genes for selecting transformed cells are already known in the literature for many organisms including different *Aspergillus*, *Rhizopus* and *Mucor*. In the event fungal expression is required, the expression system described in EP278355 (Novartis) may be thus particularly adapted.

Recombinant koji molds may be obtained by any method enabling a foreign DNA to be introduced into a cell. Such methods include transformation, electroporation, or any other technique known to those skilled in the art.

In the context of the present invention, koji molds are those traditionally used for making a koji culture including cells of the genus *Aspergillus* (ICBN taxonomy), *Rhizopus* and/or *Mucor*. Among those, the following species may be used, including *Aspergillus soyae*, *Aspergillus oryzae* (ATCC 20386), *Aspergillus phoenicis* (ATCC 14332), *Aspergillus niger* (ATCC 1004), *Aspergillus awamori* (ATCC 14331), *Rhizopus oryzae* (ATCC 4858), *Rhizopus oligosporus* (ATCC 22959), *Rhizopus japonicus* (ATCC 8466), *Rhizopus formosaensis*, *Mucor circinelloides* (ATCC 15242), *Mucor japanicus*, *Penicillium glaucum* and *Penicillium fuscum* (ATCC 10447). Strains referred by an ATCC number are accessible at the American Type Culture Collection, Rockville, Maryland 20852, US. The invention is not limited by such indications that were rather give to enable one skilled in the art to carry out the invention.

Recombinant cells of the invention may comprise the truncated *are*A gene stably integrated into the chromosome or on a replicative plasmid. Among all recombinant cells of the invention thus created, the present invention has particularly for object the strains *A. oryzae* CNCM I-1881, CNCM I-1883 and CNCM I-1884.

Preferably, only one functional truncated *are*A gene is integrated into the chromosome under the control of regulatory sequences that are native to the host organism.

In order to stably integrate into the chromosome of eucaryotic cells only one functional truncated *are*A gene which is fused to a promoter and a terminator which are native to the host organism, the DNA molecule of the invention may be integrated by slightly adapting the process of Ruiter-Jacobs *et al.* (Curr. Genet., 16, 159-163, 1989), i.e.,

(1) preparing a non-replicative DNA fragment by ligating the truncated *are*A gene, which is operably linked to a promoter and terminator that are native to the host organism, downstream the DNA sequence encoding an essential gene, said gene being inactivated by at least one mutation and/or one deletion (this essential gene may be any genes involved in RNA synthesis, such as the *pyr*G gene in case *A. oryzae* is used, for example); (2) selecting a host organism containing the essential gene which is however inactivated by another mutation(s) or deletion(s); (3)

transforming said host organism with the non replicative DNA fragment; (4) identifying integrate transformants in which the DNA fragment is integrated so as to restore the native function of the essential gene; (5) selecting an integrate transformant in which only one DNA fragment is integrated.

Over-expression of the AREA DNA-binding protein may be obtained by incorporation of the truncated *are*A gene in an expression host, said *are*A gene being operably linked to one or more regulatory sequences which serve to increase expression levels of the AREA protein of the invention.

The over-expression can be further achieved by introducing (replicative plasmid) or integrating (by integration in the genome) multiple copies of the functional truncated *are*A gene of the invention. As examples of koji molds containing multiple copies of a functional truncated *are*A genes, the transformants *Aspergilus oryzae* A (see example 1), *Aspergilus oryzae xpr*D1 (see example 3) and *Aspergilus oryzae* NF1 containing pNFF68 (see example 4) were deposited under the Budapest Treaty where they respectively receive the deposit numbers CNCM I-1881, CNCM I-1883 and CNCM I-1884.

The invention is also directed to a process for over-producing proteolytic enzymes comprising, providing koji mold of the invention in a suitable growth medium under conditions that the mold expresses proteolytic enzymes, and optionally isolating the enzymes in the form of a concentrate, for example by removing solids from the fermentation broth by centrifugation or filtration. The selection of the appropriate medium may be based on the choice of expression host and/or based on the regulatory requirements of the DNA recombinant material. Such media are well-known to those skilled in the art. After fermentation, the molds can be removed from the fermentation broth by centrifugation or filtration.

Typical L-glutamine concentrations reached during koji hydrolysis in liquid system may be 0.5-1% w/w, for example. The present koji molds are thus particularly adapted for hydrolyzing any protein containing materials, in particular those containing high amounts of L-glutamine (more than 5mM). These protein containing materials may be mixtures of a source of proteins and a source of carbohydrates, especially a mixture of a leguminous plant or of a cooked

oleaginous plant and of a cooked or roasted cereal source, for example a mixture of soya or cooked beans and of cooked or roasted wheat or rice.

Compositions containing wheat gluten are particularly adapted for the purpose of the present invention, since high amounts of L-glutamine remains sequestered in proline containing peptides when wheat gluten is hydrolyzed by traditional koji cultures.

In the event one may try, after or during hydrolysis with koji molds, to further liberate as much as possible L-glutamine linked to proline residues, the present invention provides a method in which the koji mold of the invention of the invention is used in combination with at least an enzyme or a microorganism providing a prolidase activity, that is to say an enzyme which has a high level of specificity towards dipeptides of the X-Pro type (Ezespla *et al.*, Ap. Env. Microb., 63, 314-316, 1997; Such kind of enzyme is already available from Sigma: E.C. 3.4.13.9).

In addition, the koji molds of the invention are particularly adapted for hydrolyzing protein containing materials that comprise at least 5mM of L-glutamine, allowing formation of L-glutamic acid which is an important natural taste enhancer and high degree of protein hydrolysates with excellent organoleptic properties.

In a further aspect, the present invention relates to food product comprising a protein hydrolysate obtainable by fermentation of a material comprising proteins and at least 5 mM of L-glutamine with a koji mold of the invention. Such food contains naturally high amounts of L-glutamic acid (and/or L-glutamate) and high degree of protein hydrolysates with excellent organoleptic properties leading to a non-bitter flavor and a significantly lower allergenicity than unhydrolyzed proteins

Important food product of the present invention is an ingredient of a mother milk substitute for infants, or a hydrolyzed vegetable protein ingredient. The milk substitute may be further formulated in substantially the same way as that indicated in the prior literature for products of this type (cf. EP 96202475.8).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention, in addition to those described herein, will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties to the extent necessary for understanding the present invention. DNA manipulation, cloning and transformation of bacteria cells are, except where otherwise stated, carried out according to the textbook of Sambrook *et al.* (Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, U.S.A., 1989). These examples are preceded by a brief description of the figures, of the plasmids and strains used, and by the composition of various media. The strains *A. oryzae* TK3, *Aspergilus oryzae* A (see example 1), *Aspergilus oryzae* NF2 (see example 2), *Aspergilus oryzae xpr*D1 (see example 3) and *Aspergilus oryzae* NF1 containing pNFF68 (example 4) were deposited under the Budapest Treaty, at the Collection Nationale de Culture de Microorganismes (CNCM), 25 rue du docteur Roux, 75724 Paris, France, on June 24, 1997, where they receive respectively the deposit numbers CNCM I-1882, CNCM I-1881, CNCM I-1885, CNCM I-1884, CNCM I-1883. All restrictions as to the availability of these deposits will be withdrawn upon first publication of this application or another application which claims benefit of priority to this application.

Figures

- Figure 1 shows the restriction map of pNFF21 which comprises the truncated *E. nidulans are*A gene and the *pki*A promotor and terminater.

- Figure 2 shows the relative Endo, LAP and DPPIV activities of *A. oryzae* TK3 (wild type), *A. oryzae* transformed by pNFF28 encompassing the *pyr*G gene (control *pyr*+), *A. oryzae are*A disruption mutant (control *are*A-; see example 2), and 3 mutants of *A. oryzae* NF1 which were cotransformed with pNFF28 and pNFF21.

- Figure 3 shows the restriction map of the 4.6 kb *Eco*RI-*Hind*III insert of plasmid pNFF5, which complements the *are*A19 mutation in *Emericella nidulans* G332; both exons encompassing the coding region are indicated with solid arrows.

- Figure 4 shows the *are*A disruption construct pNFF44 containing the two exons of the *E. nidulans* *pyr*G gene (*pyr*1 and *pyr*2), the two exons of *A. oryzae* *are*A gene (*are*A1 and *are*A2) and the bacterial kanamycin resistance gene (*Kana*R).

- Figure 5 shows the site directed mutagenesis of the *A. oryzae* *are*A gene; the mismatches in the mutagenic primer with the wild type *are*A sequence are indicted as follows: the stop codon (TAA) is italic, the *Afl*II site doubly underlined and the introduced *Eco*RV site is marked in bold print and is underlined.

- Figure 6 shows the relative Endo, LAP and DPPIV activities of *A. oryzae* TK3 (wild type) and 9 mutants of *A. oryzae* NF1 which were co-transformed with de-repressed *are*A amplification product and the *pyr*G amplification product. and transformants were selected on MM with glucose and glutamine.

Strains & plasmids

- *E. nidulans* G191 (*pyr*G89, *fwn*A1, *paba*A1, *Yu*A1), *E. nidulans* G353 (*are*A1, *bi*A1) and *E. nidulans* G332 (*paba*A1, *y*A2, *xpr*D1) were obtained from the Glasgow Genetic Stock Center via Dr. A.J. Clutterbuck. Other wild type strains of Emericella nidulans also may have been used in the following examples.
- *Aspergillus oryzae* TK3 was obtained from the strain collection of Nestlé.
- *Aspergillus oryzae* NF1 (*pyr*G1) is a uridine auxotroph derivative of *A. oryzae* TK3 in which the *pyr*G gene, encoding orotidine 5'-phosphate decarboxylase, was inactivated by targeted disruption.
- *Escherichia coli* BZ 234 (Collection from the Biozenter, University of Basel, Basel, Switzerland) was used as host for the propagation of plasmids. *E. coli* strains JM109 (*end*A1, *rec*A1, *gyr*A96, *hsd*R17 (r_k-, m_{k+}), *rel*A1, *sup*E44, λ^-, Δ(*lac-pro*AB), [F', *tra*D36, *pro*A^+B^+, *lac*$I^qZ\Delta M15$]) and EM1301 (*lac*Z53, *mut*S201::Tn5, *thy*A36, *rha*-5, *met*B1, *deo*C, IN(*rrn*D-*rrn*E)) were used in the site directed mutagenesis.

-The plasmid pHELP1 was used for direct cloning in *Emericella nidulans* (Gems and Clutterbuck, Curr. Genet., 24, 520-524, 1993; GenBank accession number: X78051).

- Plasmid pNFF28 contains the *A. oryzae* TK3 *pyr*G gene (GenBank accession number: Y13811).
- Plasmid pFBY182, containing the *pep*B gene as a *Eco*RI-*Xba*I fragment under the control of the *Aspergillus niger pki*A promoter and terminator was obtained from Novartis, Switzerland, via Dr. Gabor Jarai (GenBank accession number: S38698).
- pNEB193 (New England Biolabs), pAlter1 (Promega), pBluescriptSK$^-$ (Stratagene), pHSS19 and pGEM-T (Promega), and pK18 (GenBank accession number: M17626) were used for subcloning.

Media

Fungal Nitrogen Base (FNB) was composed of 1x Yeast Nitrogen Base (YNB) without amino acids and $(NH_4)_2SO_4$ (Difco) with 50 mM glucose as carbon source and 10 mM $NaNO_3$ as nitrogen source. In the case of *E. nidulans* G353 (*are*A1, *bi*A1), 10 mM glutamine was added as nitrogen source. Growth tests were performed on MM (which contains per litre 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, Pontecorvo, 1953) only now 10 mM $NaNO_3$ served as sole nitrogen source. Protease plate assays were performed on MM with 0.2% soy protein as sole carbon and nitrogen source. For quantitative studies 250 ml conical flasks filled with 80 ml of MM with 0.2% soy protein, as sole nitrogen and carbon source, were inoculated with 10^6 conidiospores/ml and incubated for 5 days at 30° C without agitation.

Exemple 1 Over-expression of the *E. nidulans* truncated *are*A gene

To assess the feasibility of increasing expression of proteolytic enzymes by modulation of *are*A expression, we decided to overexpress the *Emericella nidulans* gene in *A. oryzae* TK3.

To this end, amplification of the coding region of the *are*A gene from *Emericella nidulans* G191 and cloning of the PCR product into the expression vector pFBY182 were achieved as follows: with oligonucleotides SEQ ID NO:3 and SEQ ID NO:4, a 2.174 bp fragment, encompassing the *are*A coding region between positions 2009 and 4168, was amplified from genomic DNA of *E. nidulans* G191. At the same time an *Eco*RI site was added to 5' end and a *Xba*I site to the 3' end,

allowing directional cloning into *Eco*RI-*Xba*I digested fungal expression vector pFBY182 to give pNFF21 (see figure 1). In pNFF21, *are*A transcription is under control of the *A. niger pki*A promoter and terminator (Graaff, Curr. Genet., 22, 21-27, 1992), thereby preventing the down-regulation under repressing conditions exerted by its native 3' UTS.

pNFF21 was introduced into *A. oryzae* NF1 (*pyr*G1) by co-transformation with pNFF28 containing the *A. oryzae pyr*G gene. Accordingly, *A. oryzae* NF1 was grown in MM with 0.1% yeast extract (Difco), 50 mM glucose and 5 mM glutamine. The mycelium was harvested by sterile filtration, washed once with sterile double distilled water and once with K0.8MC (20 mM MES-HCl pH 5.8, 0.8 M KCl, 50 mM $CaCl_2$). 1.5 g of mycelium was resuspended in 20 ml of a filter sterilized 5 mg/ml solution of Novozyme 234 in K0.8MC. The mycelium suspension was incubated at 30°C for 2 hours with gentle agitation (120 rpm). The protoplasts were liberated from the mycelium by gentle resuspension with a pipet, washed twice with 20 ml of S1.0TC (10 mM Tris-HCl pH 7.5, 1 M Sorbitol, 50 mM $CaCl_2$) and were resuspended in a final concentration of 10^8/ml in S1.0TC. 20 ml of DNA was mixed with 200 µl of protoplasts and 50 µl of 25% PEG 6000 in 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$ and incubated for 20 min on ice. To this mixture, 2 ml of 25% PEG 6000 (BDH) in 10 mM Tris-HCl pH 7.5, 50 mM $CaCl_2$ were added, gently mixed and incubated for 5 min at room temperature. 4 ml of S1.0TC was added and 1.0 ml aliquots were mixed with 5 ml of 2% low melting point agarose (Sigma) in OFNB (osmotically stabilized fungal nitrogen base) and plated onto OFNB agar (Difco) with 50 mM glucose and 10 mM $NaNO_3$. *A. oryzae* NF1 transformants were plated on MM agar with 1 M sucrose, 50 mM glucose and 5 mM glutamine.

The resulting transformants were screened on MM containing 2% soy protein. Among 20 transformants screened, three showed increased secretion of proteolytic activity as judged from the sizes of the halo surrounding the colony after 36 hours of incubation at 30°C (transformants A, B and C). These three transformants were grown for five days at 30°C in stationary liquid cultures in MM with 0.2% soy protein and analyzed for proteolytic activity with the appropriate controls.

To this end, conidiospores (10^6/ml) of these three strains were used to inoculate 80 ml of liquid MM with 0.2% soy protein as sole nitrogen and carbon source. These

cultures were incubated for 5 days at 30°C without agitation. After filtration to remove the mycelium, the medium was assayed for endoproteolytic activity (Endo), Leucine aminopeptidase activity (Lap) and proline-dipeptidyl-peptidase activity (DPPIV). Endoproteolytic enzyme activity was measured with resorufin-labeled casein according to Boehringer method description supplied with the substrate (Resorufin-labeled casein, Cat.No. 1080733). Leucine aminopeptidase and dipeptidyl peptidase IV activities were determined by UV spectrometry with synthetic substrates Leu-pNa and Ala-Pro-pNa (Bachem, Switzerland), respectively, according to Sarath *et al.* (*In* Protease assay methods for proteolytic enzymes: a practical approach, Beynon R.J., Bond J.S., eds., IRL Press, Oxford). 10 mM substrate stock solution in dimethylsulfoxide (DMSO) was diluted with 100 mM sodium phosphate buffer, pH 7.0, to a final concentration of 0.5 mM. 20-100 µl culture medium supernatant was added and reaction proceeded for up to 60 min at 37°C. A control with blank substrate and blank supernatant was assayed in parallel. The release of the chromophoric group 4-nitroaniline (ε: 10'500 $M^{-1}cm^{-1}$) was measured at 400 nm and activities were expressed as mU/ml (nmol/min/ml).

Relative proteolytic activities are shown in figure 2. In the *are*A disruption mutant endoproteolytic (Endo) and leucine aminopeptidase (Lap) activity are significantly reduced compared to TK3 and the *pyr*+ control strains, whereas proline dipeptidyl peptidase activity (DPPIV) is not affected. Apparently, proline dipeptidylpeptidase expression is not under *are*A control. Introduction of multiple copies of *E. nidulans* *are*A in *A. oryzae* NF1 under the control of the *pki*A expression signals results in over-expression of endoproteolytic, leucine aminopeptidase and proline-dipeptidyl-peptidase enzyme activity.

Example 2 Over-expression of the *A. oryzae* truncated *are*A gene

1) Cloning of the *A. oryzae are*A gene: the *A. oryzae are*A gene was cloned by complementation of the corresponding *are*A gene of *E. nidulans* with the instant library method (Gems *et al.*, 1993).

First of all, the isolation of the genomic DNA was performed according to a modified protocol of the method described by Raeder and Broda (Let. appl. Microbiol., 1, 17-20, 1985). Mycelium was harvested by filtration, immediately frozen in liquid nitrogen and lyophilized. It was then reduced to a fine powder

using a mortar and pestle. 200 mg of the powdered mycelium was resuspended in 2.5 ml of extraction buffer (200 mM Tris-HCl pH 8.5 150 mM NaCl, 25 mM EDTA, 0.5 % SDS) and the solution was extracted with 1.75 ml extraction buffer-equilibrated phenol and 0.75 ml of chloroform/isoamylalcohol (24:1, v/v). The mixture was centrifuged (20 min, 3000 g). The aqueous phase was retrieved and incubated with 125 µl of RNAse A (Boehringer) solution (10 mg/ml) for 10 min at 37°C. 1.25 ml of 2-propanol (Merck) were then added. The pellet was washed with 70 % ethanol and finally resuspended in 500 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). 500 ml of 2 x QBT (1.5 M NaCl, 100 mM MOPS, 30 % ethanol, pH 7.0) were added to the sample which was then applied to a "Genomic-tip 100" (Qiagen), rinsed and eluted as recommended by the supplier.

Cloning by complementation was then achieved by mixing 40 µg *Bam*HI digested pHELP1 with either 100 µg *Bam*HI digested or 100 µg partially *Sau*3A digested genomic DNA from *A. oryzae* TK3. Additionally, 40 µg *Kpn*I digested pHELP1 was mixed with 100 µg *Kpn*I digested genomic DNA from *A. oryzae* TK3. All tree DNA mixes were introduced into *E. nidulans* G332 and transformants were selected on osmotically stabilized FNB medium with $NaNO_3$ as sole nitrogen source.

The transformation experiment with the partially digested *Sau*3A *A. oryzae* TK3 DNA, did not yield any transformants. By contrast the experiments with the *Bam*HI and *Kpn*I digested *A. oryzae* TK3 DNA did yield 14 and 3 transformants respectively. Again these transformants exhibited irregular growth, which suggested that the complementing gene was located on an autonomously replicating plasmid. In a separate experiment 40 µg *Kpn*I digested pHELP1 was co-transformed with 100 µg *Kpn*I digested genomic DNA from *E. nidulans* G332 (*xpr*D1) and one transformant was obtained.

From three *Bam*HI derived transformants and one *Kpn*I derived *are*A transformant, plasmids were rescued by transformation of *E. coli*. No plasmids could be isolated from the transformant from the *xpr*D1 transformation. From each individual *E. nidulans Bam*HI *are*A^+ transformant several plasmids could be recovered. Restriction analysis of these plasmids showed that they were pHELP1 derivatives containing additional restriction fragments, but that not all of these inserts carried terminal *Bam*HI sites. Similarly, from the *Kpn*I *are*A^+ transformant

several pHELP1 derivatives could be recovered, non of which had an insert with terminal *Kpn*I sites. These observations indicate instability of the plasmids

One *Bam*HI (pNFF3) and one *Kpn*I (pNFF4) pHELP1 derivative were chosen for further analysis. The inserts of both clones hybridized to the coding region of the *E. nidulans* *are*A gene. Detailed analysis of these two clones showed that in pNFF3, the entire *are*A gene was located on a 4.6 kb *Eco*RI-*Hind*III fragment (Fig. 3). This 4.6 kb *Eco*RI-*Hind*III fragment was subcloned into pHSS19 to give pNFF5. Upon re-introduction into *E. nidulans* G323, pNFF5 restores its ability to grow on $NaNO_3$ as sole nitrogen source demonstrating that this plasmid contains a functional *are*A gene (data not shown).

2) Characterization of the *A. oryzae are*A gene: the complete nucleotide sequence of the *Eco*RI-*Hind*III insert of pNFF5 was determined by analysis of both strands on partially overlapping subclones. The nucleotide sequence was determined, on a Licor model 4000 automatic sequencer. IRD41 labeled primers were used for sequencing both strands of partially overlapping subclones by the dideoxynucleotide method of Sanger *et al.* (Proc Natl Acad Sci USA, 74, 5463-5467, 1977). The DNA sequence analysis was performed by using the GCG Computer programs (Devereux *et al.*, Nucl. Acids Res., 12, 387-395, 1987).

Results show that the *A. oryzae are*A gene encodes a protein of 853 amino acid residues with a deduced molecular weight of 91.5 kDa (see SEQ ID NO:2). At the protein level the *A. oryzae are*A exhibits a similarity of 83% and at the DNA level 70% similarity with the *E. nidulans are*A gene.

Moreover, in the putative promoter region the overall DNA homology with *E. nidulans* drops to 43%. Still, seven stretches of DNA 5 to 13 bp long show 100% sequence identity and occupy virtually identical positions in both promoters. These sequences could represent *cis*-acting elements. Additionally, the 5' non-transcribed region contains several putative AREA-binding sites (GATA or TATC; Fu and Marzluf, Proc. Natl. Acad. Sci USA, 87, 5351-5355, 1990) two of which occupy identical positions as the two functional AREA-binding sites in *E. nidulans*.

3) Disruption of the *A. oryzae are*A gene: to elucidate the role of *are*A in the expression of protease encoding genes, an *are*A-null mutant was generated by

gene disruption. To construct such an *are*A null allele, the two internal *Sma*I fragments (see Fig. 3) were removed from pNFF5 to give pNFF10. To do so, pNFF10 was created by digesting pNFF5, containing the *A. oryzae* TK3 *are*A gene, with *Sma*I and selfligating the vector containing fragment. This deleted the internal 0.5 and 0.2 kb *Sma*I fragments from the second exon of the *are*A gene in pNFF5

As selection marker, a PCR product, encompassing the *E. nidulans* *pyr*G gene, was inserted into the unique *Sma*I site of pNFF10 to give pNFF44 (Fig.4). Accordingly, with oligonucleotides SEQ ID NO:5 and SEQ ID NO: 6 the *pyr*G gene was amplified from *E. nidulans* G332 and the 1.851 bp PCR product cloned into pGEM-T (Promega) to give pNFF38 and pNFF39. The *Eco*RI fragment, encompassing the *pyr*G gene was retrieved from pNFF39, blunt ended with T4 DNA polymerase and cloned into the *Sma*I site of pNFF10.

This pNFF44 construct, linearized with *Eco*RI and *Nhe*I, was used to transform *A. oryzae* NF1, and transformants were selected on osmotically stabilized MM containing glucose and glutamine as carbon and nitrogen source respectively. All *pyr*G$^+$ transformants were further checked for their ability to use nitrate and soy protein as sole nitrogen sources. Four *pyr*G$^+$ transformants exhibited greatly reduced or no growth on nitrate MM and three did not form a halo when grown for two days on MM containing 0.2% soy protein as sole nitrogen and carbon source (data not shown). A Southern blot of *Sma*I digested genomic DNA of these four and six other *pyr*G$^+$ transformants was digested with *Sma*I and probed with the 4.6 kb *Eco*RI-*Hind*III insert of pNFF5. Only in one of the transformants the two internal *Sma*I fragments of the *are*A gene were deleted, identifying this transformant as an *are*A null-mutant. This *are*A disruption mutant was called NF2.

The *are*A mutant NF2 was grown for 5 days at 30°C without agitation in 80 ml of MM with 0.2% soy protein. The *are*A mutant grew poorly on MM with 0.2% soy protein. Analysis of the culture broth showed a 75% decrease in total endoproteolytic activity and a 60% decrease in leucine aminopeptidase activity compared to the *A. oryzae* TK3 (WT) control (Fig 2). By contrast the proline dipeptidylpeptidase activity in the *are*A mutant did not significantly differ from the wild type control (Fig. 2) .

4) Construction of a constitutive *are*A allele : co-transformation experiments with pNFF5, containing the WT *are*A gene, did not yield co-transformants that overproduced proteolytic enzymes (data not shown). This suggested tight regulation of the *A. oryzae are*A gene.

To allow the constitutive expression of proteolytic enzymes (i.e. in the presence of glutamine), truncation of the *are*A gene was achieved. By site directed mutagenesis, a stop codon (TAA), an *Afl*II and an *Eco*RV site were introduced into the 4.6 kb *Eco*RI-*Hind*III *are*A fragment, truncating the AREA protein after amino acid residue 752 (see figure 5).

To this end, the *Eco*RI-*Hind*III insert of pNFF5 was ligated into pALTER1 and introduced into *E. coli* JM109 to give pNFF49. By superinfection with the helperphage M13KO7, single stranded DNA was generated from pNFF49 which was used in the site directed mutagenesis procedure with the Altered sites II kit (Promega). Then 0.05 pmol single stranded pNFF49 was annealed to 0.25 pmol Ampicillin repair oligonucleotide SEQ ID NO:7, 0.25 pmol Tetracycline knock-out oligonucleotide SEQ ID NO: 8 and 1.25 pmol *are*A/*xpr*D1 mutagenic oligonucleotide SEQ ID NO:9, in 20 ml of 20 mM Tris-HCl pH 7.5,10 mM $MgCl_2$ and 50 mM NaCl in a Perkin Elmer Thermocycler programmed to heat the annealing mixture to 75°C for 5 min and then to cool to 45° C at a rate of 1°C/min. From 45°C to 20° the cooling rate was increased to 1.5°C/min. Next 3 ml 100 mM Tris-HCl pH 7.5, 5 mM dNTPs, 10 mM ATP and 20 mM DTT were added. The mixture was incubated for 90 min at 37°C with 5U T4 DNA polymerase and 1U T4 DNA ligase. A 3 ml aliquot of the reaction mixture was introduced into *E. coli* ES1301 by electroporation and transformants were selected in 5 ml LB containing 125 mg/ml ampicillin. The mutagenised plasmids were recovered from ES1301 and introduced into BZ234.

The 3.5 kb *Eco*RI-*Eco*RV fragment was further cloned into pBlueskript to give pNFF58. To test functionality pNFF58 was introduced into *A. oryzae* NF2 (see above) and transformants were selected on OFNB containing $NaNO_3$ as sole nitrogen source. With pNFF58, 1.5 transformants/µg were obtained and with the control pNFF5, 6 transformants/µg. These data prove that pNFF58 still contains a functional *are*A gene. The pNFF58 transformants were screened for proteolytic activity on MM with 0.2% soy protein and MM with 0.2% soy protein and 10 mM

glutamine. On 0.2% soy protein several transformants produced bigger halos that the wild type control (*A. oryzae* TK3) suggesting that overexpression results in enhanced secretion of proteolytic enzymes. Most transformants produced halos on both media, suggesting derepressed expression of proteolytic enzymes (data not shown).

Example 3 Construction of protease-overproducing Koji mould strains.

In order to produce potential production koji mold strains, at least one additional copy of the de-repressed *are*A allele would need to be introduced into the *A. oryzae* TK3 derivative NF1. For legal reasons, this had to be done without introducing bacterial sequences, especially antibiotic resistance genes. To this end the inserts of pNFF28 and pNFF58 were amplified by PCR with *Pfu*I DNA polymerase and phosphorylated oligonucleotides SEQ ID NO:10 and SEQ ID NO:11. The amplification products were selfligated and purified. 10 µg of the pNFF58 amplification product and 10 µg of the pNFF28 amplification product were introduced into *A. oryzae* NF1 and the transformants were selected on osmotically stabilised MM with 50 mM glucose and 5 mM glutamine. As a control also 10 µg of pNFF28 was introduced. The plasmid pNFF28 yielded 30 transformants/µg, the pNFF28 PCR product 6 transformants/µg and the pNFF28/pNFF58 PCR products 16 transformants/µg.

The potential co-transformants were screened for increased protease activity on MM with 0.2% soy protein and MM with 0.2% soy protein and 10 mM L-glutamine. Twelve transformants produced more proteolytic activity on both media as indicated by the increased size of the halo they produced. To quantify the overexpression, nine of them were incubated without agitation for 5 days at 30°C in 80 ml MM containing 0.2% soy protein. The culture media were assayed for proteolytic activity (Fig. 6).

As with the *E. nidulans are*A gene under control of the *A. niger pki*A expression signals (Fig. 2) all three classes of proteolytic activity tested were increased compared to the *A. oryzae* TK3 wild type and a *pyr*G$^+$ derivative of *A. oryzae* NF1.

Southern analysis of the protease overproducing strains showed that all co-transformants contain 2 to 4 functionally integrated copies of the de-repressed *areA* gene.

Comparing the observed levels of protease overproduction and the number of functionally integrated copies of de-repressed *are*A gene, no clear relation was observed. Transformant *xpr*D1 produces the highest level of proteolytic activity and contains multiple copies of functionally integrated *xpr*D1. However, transformant *xpr*D12 contains far less copies of functionally integrated *xpr*D1 but produces almost as much activity as transformant *xpr*D1. Furthermore, the hybridisation patterns of *xpr*D6 and *xpr*D7 are very similar, yet *xpr*D6 overproduces all activities tested 1.5 fold but *xpr*D7 overproduces only proline dipeptidylpeptidase.

Example 4 Expression of *A. oryzae xpr*D1 allele with the promoter and terminater of the *A. oryzae dpp*IV gene

Co-transformation experiments of example 2 resulted in strains that had muliple copies of pNFF58 integrated in the genome and that overproduced proteolytic activity 2 to 3 fold when compare to the wild type TK3 strain. By contrast, strains with one copy of pNFF21 (example 1), where *E. nidulans* areA is under the control of a strong glycolytic promoter resulted in 6 fold over-expression. These data suggest that the native *are*A promoter is a weak promoter and that expression of a functional truncated *are*A under control of a strong promoter gives better results.

To this end, the *dpp*IV gene of *A. oryzae* TK3 was amplified by PCR with *Pfu*I DNA polymerase and phosphorylated oligonucleotides SEQ ID NO:12 and SEQ ID NO:13. The PCR product was then digested with *Apa*I and *Eco*RV enzymes. The digested *Apa*I-*Eco*RV 4.8 kb fragment was subcloned into pALTER1 (Promega) to give pNFF61. Next pNFF61 was subjected to a site directed mutagenesis according to the protocol of Deng *et al.* (Anal. Biochem., 200, 81, 1992), using the 5'-phosphorylated mutagenic oligonucleotides SEQ ID NO:14 and SEQ ID NO:15 according to the manual with Altered sites II kit (Promega) resulting in pNFF62. Using the polymerase enzyme *Pfu*I and the oligonuclotides SEQ ID NO:16 and SEQ ID NO:17, the *xpr*D1 allele was amplified by PCR, from pNFF58 containing the *A. oryzae xpr*D1 allele, as a 3.4 kb *Eco*RI-*Eco*RV

fragment. The 2294 bp *xpr*D1 amplification product was then phosphorylated and cloned into the *Sma*I digested vector pK19 (Pridmore *et al.*, Gene, 56, 309-312, 1987) to give pNFF64. Finally the *Not*I-*Ecl*136III insert from pNFF64 was inserted into *Not*I-*Hpa*I pNFF62 creating pNFF68 encompassing the *xpr*D1 allele fused to the *dpp*IV promoter and terminater.

PNFF68 was intoduced into A. oryzae NF1 by co-transformation with pNFF28, and primary transformants were screened for increased proteolytic activity on MM plates containing 0.2% soy protein. Five out of 35 transformants exhibited increased halo sizes compared to *A. oryzae* TK3. Among the 5 transformants thus selected, one was deposited under the Budapest Treaty at the CNCM, where it receives the deposit number CNCM I-1883.

Co-transformants over-expressing proteolytic enzymes and wild type controls were plated on MM plates containing 0.2% soy protein and 5 mM L-glutamine. All the selected co-transformants still produced a halo in the presence of 5 mM glutamine, whereas the wild type did not, indicating de-repressed expression of proteolytic activity.

To quantify the over-expression, transformants were incubated without agitation for 5 days at 30°C in 80 ml MM containing 0.2% soy protein. The culture media were then assayed for proteolytic activity. Results show an overproduction of proteolytic activity of at least 6 fold when compare to the wild type TK3 strain.

Examples 5

For preparing a fermented soya sauce, a koji is prepared by mixing an *Aspergillus oryzae* CNCM I-1883 koji culture with a mixture of cooked soya and roasted wheat, the koji is then hydrolyzed in aqueous suspension for 3 to 8 hours at 45°C to 60°C with the enzymes produced during fermentation of the *Aspergillus oryzae* CNCM I-1 culture, a moromi is further prepared by adding suitable amount of sodium chloride to the hydrolyzed koji suspension, the moromi is left to ferment and is then pressed and the liquor obtained is pasteurized and clarified.

Examples 6

For producing a flavouring agent, a aqueous suspension of a mixture of cooked soya and roasted wheat is prepared, the proteins are solubilized by hydrolysis of the suspension with a protease at pH6.0 to 11.0, the suspension is heat-trated at pH 4.6 to 6.5, and the suspension is ripened with the prolidase enzyme of Sigma and proteolytic enzymes which have been isolated from a liquid medium fermented by *Aspergillus oryzae* CNCM I-1881.

SEQUENCE LISTING

```
(1) GENERAL INFORMATION:
    (i)   APPLICANT:
    (ii)  (A) NAME: SOCIETE DES PRODUITS NESTLE
          (B) STREET: AVENUE NESTLE 55
          (C) CITY: VEVEY
          (D) STATE: VAUD
          (E) COUNTRY: SWITZERLAND
          (F) POSTAL CODE (ZIP): 1500
    (ii) TITLE OF INVENTION: ENHANCED EXPRESSION OF PROTEOLYTIC ENZYMES
         IN KOJI MOLDS
   (iii) NUMBER OF SEQUENCES: 17
    (iv) COMPUTER READABLE FORM:
          (A) MEDIUM TYPE: Floppy disk
          (B) COMPUTER: IBM PC compatible
          (C) OPERATING SYSTEM: PC-DOS/MS-DOS
          (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:
    (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 4657 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: double
          (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: DNA (genomic)
    (ix) FEATURE:
          (A) NAME/KEY: exon
          (B) LOCATION:1189..1604
    (ix) FEATURE:
          (A) NAME/KEY: intron
          (B) LOCATION:1605..1703
    (ix) FEATURE:
          (A) NAME/KEY: exon
          (B) LOCATION:1704..3846
    (ix) FEATURE:
          (A) NAME/KEY: misc_feature
          (B) LOCATION:1189..3480
          (D) OTHER INFORMATION:/label= TRUNCATED-AREA
                               /note = "AREA IS TRUNCATED IMMEDIATELY
                                       DOWNSTREAM THE SEQUENCE ENCODING
                                       A DNA BINDING DOMAIN"
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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AATCGCACTT CTTTTCTTAT TTCCTTGTGG GATAAATTGA TTATTTACCG TTTCGTTTTC 720

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 853 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(ix) FEATURE:
(A) NAME/KEY: Binding-site
(B) LOCATION: 652-676
(D) OTHER INFORMATION:/note= "DNA BINDING SITE"

(ix) FEATURE:
(A) NAME/KEY: Region
(B) LOCATION:1..731
(D) OTHER INFORMATION:/note= "TRUNCATED AREA WHICH IS STILL ACTIVE BUT NOT REPRESSED BY L-GLUTAM..."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Gln Thr Ala Thr Phe Thr Thr His His Pro Ser Ala Asp Ala Asp Arg
            20                  25                  30

Pro Asn Asn Leu Pro Pro Thr Ser Ser Gln Leu Ser Asp Asp Phe Ser
        35                  40                  45

Phe Gly Ser Pro Leu Ser Pro Ala Asp Ser Gln Ala His Asp Gly Leu
    50                  55                  60

Leu Gln Asp Ser Leu Phe Pro Glu Trp Gly Ser Gly Ala Pro Arg Pro
65                  70                  75                  80

Gly Ile Asp Ser Pro Asp Glu Met Gln Arg Gln Asp Pro Leu Ala Thr
                85                  90                  95

Gln Ile Trp Lys Leu Tyr Ser Arg Thr Lys Ala Gln Leu Pro Asn Gln
            100                 105                 110

Glu Arg Met Glu Asn Leu Thr Trp Arg Met Met Ala Met Ser Leu Lys
        115                 120                 125

Arg Lys Glu Arg Glu Arg Ala Gln Gln Ser Ile Gly Ile Ala Gln Leu
    130                 135                 140
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Arg Ile Ser Asp Pro Pro Val Ala Thr Gly Asn Pro Gln Ser Thr Asp
145                 150                 155                 160

Leu Thr Ala Asp Pro Met Asn Leu Asp Asp Phe Ile Val Pro Phe Glu
                165                 170                 175

Ser Pro Ser Asp His Pro Ser Pro Ser Ala Val Lys Ile Ser Asp Ser
            180                 185                 190

Thr Ala Ser Ala Ala Ile Pro Ile Lys Ser Arg Lys Asp Gln Leu Arg
        195                 200                 205

Asp Ser Thr Pro Val Pro Ala Ser Phe His His Pro Ala Gln Asp Gln
    210                 215                 220

Arg Lys Asn Ser Glu Phe Gly Tyr Val Pro Arg Arg Val Arg Lys Thr
225                 230                 235                 240

Ser Ile Asp Glu Arg Gln Phe Phe Ser Leu Gln Val Pro Thr Arg Lys
                245                 250                 255

Arg Pro Ala Glu Ser Ser Pro Gln Val Pro Pro Val Ser Asn Ser Met
            260                 265                 270

Leu Ala His Asp Pro Asp Leu Ala Ser Gly Val Pro Asp Tyr Ala Leu
        275                 280                 285

Asp Ala Pro Ser Ser Ala Phe Gly Phe His Gln Gly Asn His His Pro
    290                 295                 300

Val Asn His His Asn His Thr Ser Pro Gly Ala Pro Phe Gly Leu Asp
305                 310                 315                 320

Thr Phe Gly Leu Gly Asp Asp Pro Ile Leu Pro Ser Ala Gly Pro Tyr
                325                 330                 335

Gln Ser Gln Phe Thr Phe Ser Pro Ser Glu Ser Pro Met Ala Ser Gly
            340                 345                 350

His Pro Phe Ala Asn Leu Tyr Ser His Thr Pro Val Ala Ser Ser Leu
        355                 360                 365

Asn Ser Thr Asp Phe Phe Ser Pro Pro Pro Ser Gly Tyr Gln Ser Thr
    370                 375                 380

Ala Ser Thr Pro Gln Pro Thr Tyr Asp Gly Asp His Ser Val Tyr Phe
385                 390                 395                 400

Asp Met Pro Ser Gly Asp Ala Arg Thr Gln Arg Arg Ile Pro Asn Tyr
                405                 410                 415

Ile Ser His Arg Ser Asn Leu Ser Ala Ser Leu Gln Pro Arg Tyr Met
            420                 425                 430

Phe Asn Gln Asn Asn His Glu Gln Ala Ser Ser Ser Thr Val His Ser
        435                 440                 445

Pro Ser Tyr Pro Ile Pro Gln Pro Gln His Val Asp Pro Thr Gln Val
    450                 455                 460

Leu Asn Ala Thr Asn Tyr Ser Thr Gly Asn Ser His His Thr Gly Ala
465                 470                 475                 480

Met Phe Ser Phe Gly Ala Asp Ser Asp Asn Glu Asp Gly Asp Gly His
                485                 490                 495

Gln Leu Ser Glu Arg Ala Gly Leu Ala Met Pro Thr Glu Tyr Gly Asp
            500                 505                 510
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Glu Asp Gly Phe Ser Ser Gly Met Gln Trp Asp Gly Gln Phe Pro Gly
        515                 520                 525

Ser Phe His Ser Leu Pro Gly Phe Gly Pro Gln His Arg Lys His Val
    530                 535                 540

Thr Ile Gly Ser Thr Asp Met Met Asp Thr Pro Glu Glu Trp Asn His
545                 550                 555                 560

Gly Gly Ser Leu Gly Arg Thr His Gly Ser Val Ala Ser Val Ser Glu
                565                 570                 575

Val Arg Asn Arg Glu Gln Asp Pro Arg Arg Gln Lys Ile Ala Arg Thr
            580                 585                 590

Thr Ser Thr Pro Asn Thr Ala Gln Leu Leu Arg Gln Ser Met His Ser
        595                 600                 605

Asn Asn Asn Thr Ser His Thr Ser Pro Asn Thr Pro Pro Glu Ser Ala
    610                 615                 620

Leu Ser Ser Ala Val Pro Ser Arg Pro Ala Ser Pro Gly Gly Ser Lys
625                 630                 635                 640

Asn Gly Asp Gln Gly Ser Asn Gly Pro Thr Thr Cys Thr Asn Cys Phe
                645                 650                 655

Thr Gln Thr Thr Pro Leu Trp Arg Arg Asn Pro Glu Gly Gln Pro Leu
            660                 665                 670

Cys Asn Ala Cys Gly Leu Phe Leu Lys Leu His Gly Val Val Arg Pro
        675                 680                 685

Leu Ser Leu Lys Thr Asp Val Ile Lys Lys Arg Asn Arg Ser Ser Ala
    690                 695                 700

Asn Ser Leu Ala Val Gly Thr Ser Arg Ala Ser Lys Lys Thr Ala Arg
705                 710                 715                 720

Lys Asn Ser Val Gln Gln Ala Ser Val Thr Thr Pro Thr Ser Ser Arg
                725                 730                 735

Ala Gln Asn Gly Thr Ser Glu Ser Pro Pro Ala Gly Phe Ser Ala Ala
            740                 745                 750

Ala Gly Arg Ser Asn Gly Val Val Pro Ile Ala Ala Ala Pro Pro Lys
        755                 760                 765

Ala Ala Pro Ser Ala Ala Ala Ser Pro Ser Thr Gly Gln Thr Arg Asn
    770                 775                 780

Pro Ile Gln Ala Ala Pro Lys Arg Gln Arg Arg Leu Glu Lys Ala Thr
785                 790                 795                 800

Glu Met Glu Thr Asp Glu Ala Asn Lys Ser Ala Gly Gly Arg Ser Lys
                805                 810                 815

Val Val Pro Leu Ala Pro Ala Met Pro Pro Ala Ala Ala Asn Pro Ala
            820                 825                 830

Asn His Ser Ile Ala Gly Gly Gln Gly Ala Ser Gln Glu Trp Glu Trp
        835                 840                 845

Leu Thr Met Ser Leu
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(2) INFORMATION FOR SEQ ID NO: 3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGAATTCATG AGTGGCATCG C 21

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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(2) INFORMATION FOR SEQ ID NO: 6:
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(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(2) INFORMATION FOR SEQ ID NO: 7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GTTGCCATTG CTGCAGGCAT CGTGGTG 27

(2) INFORMATION FOR SEQ ID NO: 8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GCCGGGCCTC TTGCGGGCGT CCATTCC 27

(2) INFORMATION FOR SEQ ID NO: 9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CATCCGTCAC GACTTAAGAT ATCAAGCCGC GC 32

(2) INFORMATION FOR SEQ ID NO: 10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CACAGGAAAC AGTCACGAC 19

(2) INFORMATION FOR SEQ ID NO: 11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CGTTTTCCCA GTCACGAC 18

(2) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GGGCCCGGTA CCCAATTCGC CC 22

(2) INFORMATION FOR SEQ ID NO: 13:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GATATCGGTT TATTGTGGCC G 21

(2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

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(2) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GACTTGGAGG AGTAGTTAAC GGCACATCAT TC 32

(2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATGCGGCCGC TAACCCTCGG GCGAGGCCC 29

(2) INFORMATION FOR SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "OLIGONUCLEOTIDE"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TTAAGTCGTG ACGGATGCTT GC 22

Claims

1. A koji mold which is capable to express at least 2 times more endo- and exo-peptidases than the wild type strain *Aspergillus oryzae* CNCM I-1882.

2. A koji mold according to claim 1, which expresses at least 30 mU of endopeptidase activity, at least 30 mU of leucine-amino-peptidase activity and at least 10 mU of proline-dipeptidyl-peptidase activity per ml of supernatant when grown in a minimal medium containing 0.2% soy bean proteins.

3. A koji mold according to claim 1, which is capable to express the proteolytic activities in presence of at least 5mM L-glutamine.

4. A koji mold according to claim 1, which contains an *are*A gene which is not repressed when the mold is grown in a minimal medium containing repressive amounts of L-glutamine.

5. A koji mold according to claim 4, wherein the *are*A gene is truncated so the C-terminally truncated AREA protein remains functional but not not repressed when the mold is grown in a minimal medium containing repressive amounts of L-glutamine.

6. A koji mold according to claim 4, which has integrated multiple copies of the *are*A gene.

7. A koji mold according to claim 5, wherein the *are*A gene is operably linked to at least one regulatory sequence able to direct over-expression of the *are*A gene.

8. A koji mold according to claims 5 or 6, wherein the *are*A gene has the nucleotide sequence defined by nucleotides 1189-1604 and 1704-3480 of SEQ ID NO:1 or functional derivatives thereof due to the degeneracy of the genetic code.

9. A koji mold according to one of any preceeding claims 1-8 selected from the genus *Aspergillus, Rhizopus* or *Mucor*.

10. A koji mold according to claim 9 which is selected from strains *Aspergillus oryzae* CNCM I-1881, CNCM I-1883 and CNCM I-1884.

11. A DNA-binding protein of *Aspergillus oryzae* (AREA) having at least the amino-acid sequence from amino-acid 1 to amino-acid 731 of SEQ ID NO:2 or functional derivatives thereof.

12. A DNA molecule which comprises an *are*A gene encoding the protein according to claim 11.

13. A DNA molecule according to claim 12, which is a vector comprising the *are*A gene.

14. A DNA molecule according to claim 12, wherein the *are*A gene is operably linked to at least one regulatory sequence able to direct the expression of the said gene.

15. A DNA molecule according to claim 12, wherein the *are*A gene has at least the nucleotide sequence defined by nucleotides 1189-1604 and 1704-3480 of SEQ ID NO:1 or functional derivatives thereof due to the degeneracy of the genetic code.

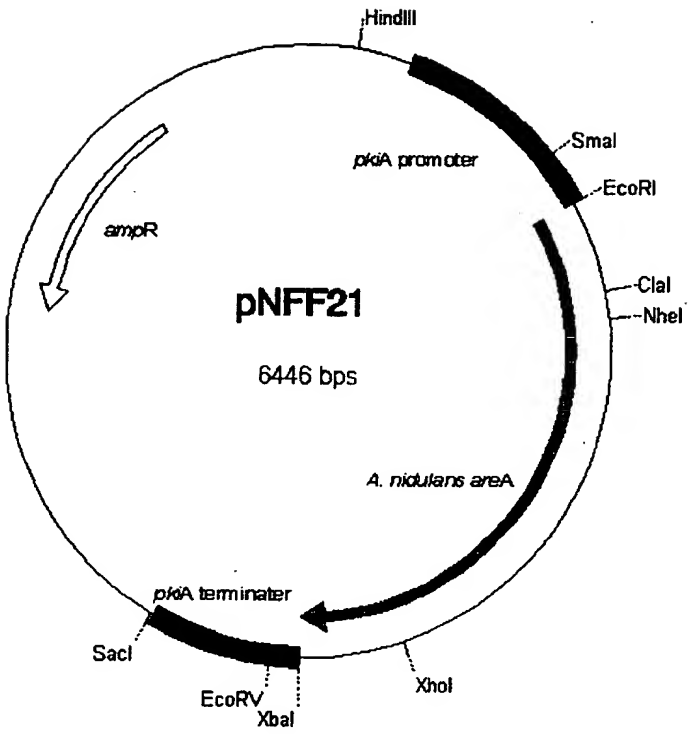
16. A method for over-producing proteolytic enzymes, comprising cultivating a koji mold according to claims 1-10 in a suitable growth medium under conditions that the mold expresses enzymes, and optionally isolating the enzymes in the form of a concentrate.

17. Use of the koji mold according to claim 1-10 to hydrolyse protein-containing materials.

18. Use according to claim 17, in combination with an enzyme and/or a microoganisme providing a prolidase activity.

19. Use according to claims 17 or 18, wherein the protein-containing materials comprise at least 5mM of L-glutamine.

20. A food product comprising a protein hydrolysate obtainable by fermentation with a koji mold according to claims 1-10 of a material comprising proteins and at least 5mM of L-glutamine.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SmaI
pkiA promoter
EcoRI
ampR
ClaI
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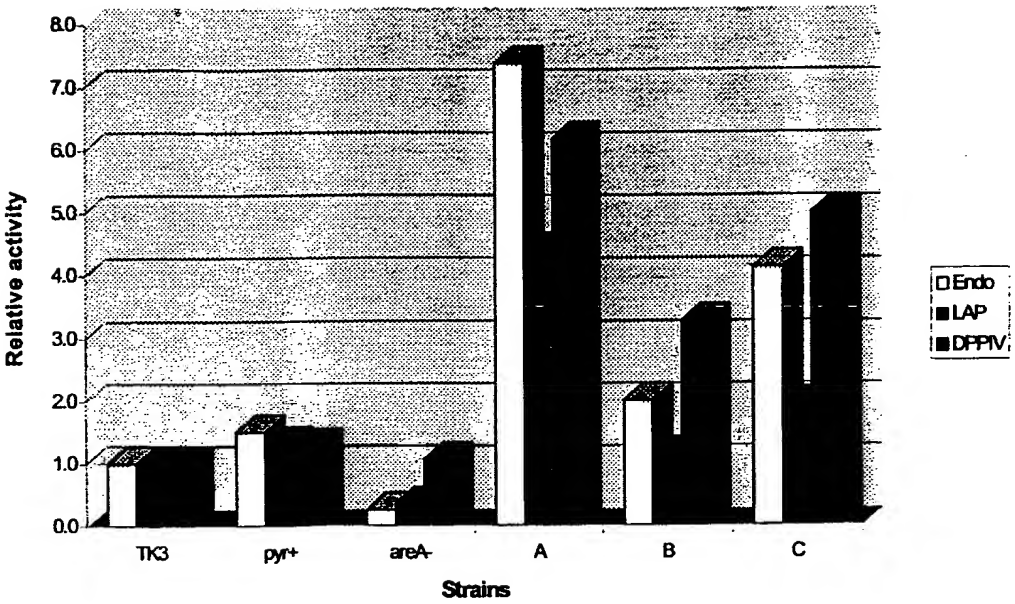
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B
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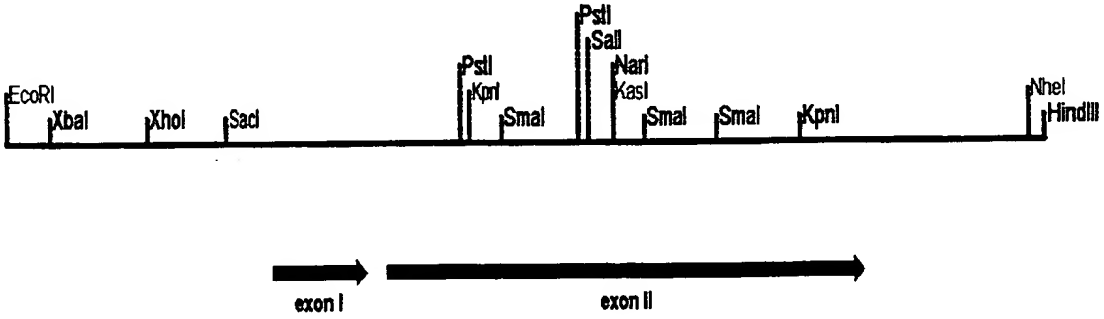
Endo
LAP
DPPIV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PstI
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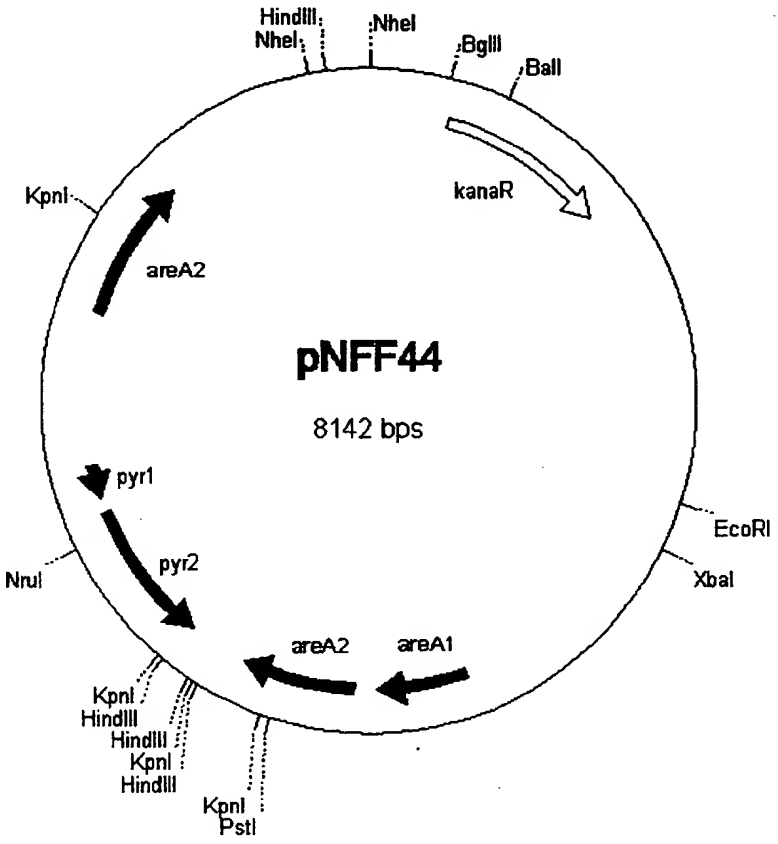
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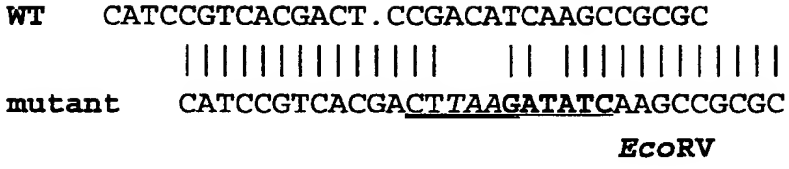
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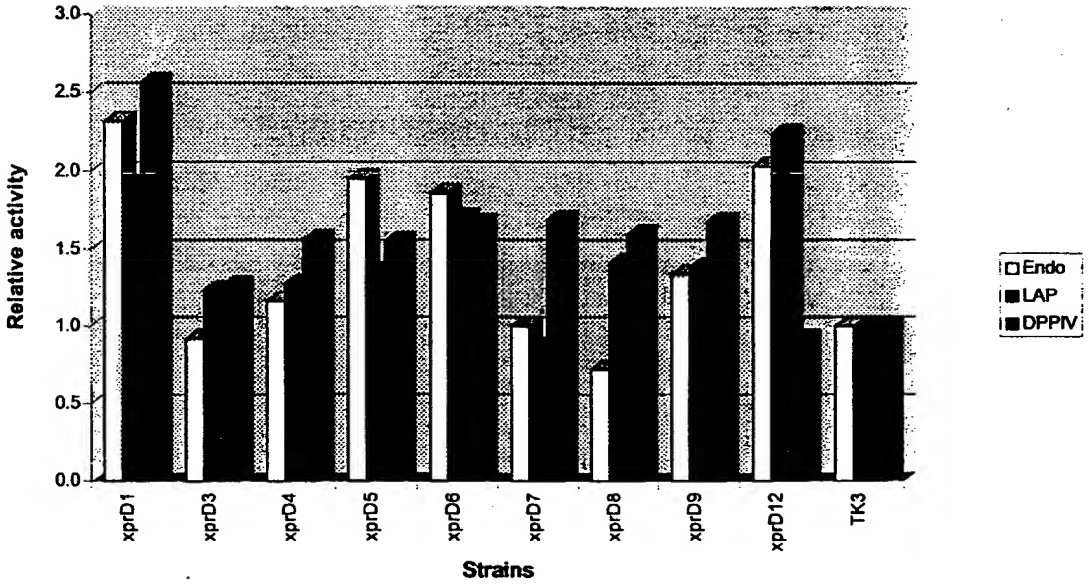
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xprD6
xprD7
xprD8
xprD9
xprD12
TK3
Strains
Endo
LAP
DPPIV

TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

DESTINATAIRE :

SOCIETE DES PRODUITS NESTLE S.A.
Département des Brevets
Avenue Nestlé 55
CH-1800 VEVEY

NOM ET ADRESSE
DU DEPOSANT

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
L'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :
Aspergillus oryzae strain A (NO 5996/GF)	I - 1881

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le 24 JUIN 1997 (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom : CNCM
Collection Nationale
de Cultures de Microorganismes

Adresse : INSTITUT PASTEUR
28, Rue du Docteur Roux
F-75724 PARIS CEDEX 15

Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : Mme Y. CERISIER
Directeur Administratif de la CNCM

Date : Paris, le 14 octobre 1997

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
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FORMULE INTERNATIONALE

DESTINATAIRE :

SOCIETE DES PRODUITS NESTLE S.A.
Département des Brevets
Avenue Nestlé 55
CH-1800 VEVEY

RECEPISSE EN CAS DE DEPOT INITIAL, délivré en vertu de la règle 7.1 par L'AUTORITE DE DEPOT INTERNATIONALE identifiée au bas de cette page

NOM ET ADRESSE DU DEPOSANT

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :
Aspergillus oryzae TK3 (NO 5861/GF)	**I - 1882**

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **24 JUIN 1997** (date du dépôt initial) [1]

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et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom : **CNCM** Collection Nationale de Cultures de Microorganismes Adresse : **INSTITUT PASTEUR** 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : Mme Y. CERISIER, Directeur Administratif de la CNCM Date : Paris, le 14 octobre 1997

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TRAITE DE BUDAPEST SUR LA RECONNAISSANCE
INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
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DESTINATAIRE :

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Département des Brevets
Avenue Nestlé 55
CH-1800 VEVEY

NOM ET ADRESSE
DU DEPOSANT

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
L'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :

Aspergillus oryzae containing pNFF68

Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :

I - 1883

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

☐ d'une description scientifique

☐ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **24 JUIN 1997** (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

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Avenue Nestlé 55
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NOM ET ADRESSE
DU DEPOSANT

RECEPISSE EN CAS DE DEPOT INITIAL, délivré en vertu de la règle 7.1 par L'AUTORITE DE DEPOT INTERNATIONALE identifiée au bas de cette page

I. IDENTIFICATION DU MICRO-ORGANISME	
Référence d'identification donnée par le DEPOSANT : **Aspergillus oryzae xprD1 (NO 5996/GF)**	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE : **I - 1884**

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le **24 JUIN 1997** (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

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V. AUTORITE DE DEPOT INTERNATIONALE

Nom : **CNCM** Collection Nationale de Cultures de Microorganismes	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) : Mme Y. CERISIER, Directeur Administratif de la CNCM
Adresse : **INSTITUT PASTEUR** 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15	Date : Paris, le 14 octobre 1997

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

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INTERNATIONALE DU DEPOT DES MICRO-ORGANISMES
AUX FINS DE LA PROCEDURE EN MATIERE DE BREVETS

FORMULE INTERNATIONALE

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Département des Brevets
Avenue Nestlé 55
CH-1800 VEVEY

NOM ET ADRESSE
DU DEPOSANT

RECEPISSE EN CAS DE DEPOT INITIAL,
délivré en vertu de la règle 7.1 par
L'AUTORITE DE DEPOT INTERNATIONALE
identifiée au bas de cette page

I. IDENTIFICATION DU MICRO-ORGANISME

Référence d'identification donnée par le DEPOSANT :	Numéro d'ordre attribué par l'AUTORITE DE DEPOT INTERNATIONALE :
Aspergillus oryzae NF2 (NO 5996/GF)	I - 1885

II. DESCRIPTION SCIENTIFIQUE ET/OU DESIGNATION TAXONOMIQUE PROPOSEE

Le micro-organisme identifié sous chiffre I était accompagné :

■ d'une description scientifique

■ d'une désignation taxonomique proposée

(Cocher ce qui convient)

III. RECEPTION ET ACCEPTATION

La présente autorité de dépôt internationale accepte le micro-organisme identifié sous chiffre I, qu'elle a reçu le 24 JUIN 1997 (date du dépôt initial) [1]

IV. RECEPTION D'UNE REQUETE EN CONVERSION

La présente autorité de dépôt internationale a reçu le micro-organisme identifié sous chiffre I le (date du dépôt initial)
et a reçu une requête en conversion du dépôt initial en dépôt conforme au Traité de Budapest le (date de réception de la requête en conversion)

V. AUTORITE DE DEPOT INTERNATIONALE

Nom :	Signature(s) de la (des) personne(s) compétente(s) pour représenter l'autorité de dépôt internationale ou de l'(des) employé(s) autorisé(s) :
CNCM Collection Nationale de Cultures de Microorganismes	Mme Y. CERISIER Directeur Administratif de la CNCM
Adresse : INSTITUT PASTEUR 28, Rue du Docteur Roux F-75724 PARIS CEDEX 15	Date : Paris, le 14 octobre 1997

1 En cas d'application de la règle 6.4.d), cette date est la date à laquelle le statut d'autorité de dépôt internationale a été acquis.

Formule BP/4 (page unique)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/02785

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/31 C12N1/15 C07K14/38 C12N9/62 A23J3/16
A23J3/18 C12P21/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUDLA B ET AL: "THE REGULATORY GENE AREA MEDIATING NITROGEN METABOLITE REPRESSION IN ASPERGILLUS NIDULANS. MUTATIONS AFFECTING SPECIFICITY OF GENE ACTIVATION ALTER A LOOP RESIDUE OF A PUTATIVE ZINC FINGER" EMBO JOURNAL, vol. 9, no. 5, April 1990, pages 1355-1364, XP000615427 cited in the application	1-15
Y	see the whole document especially figure 9	16-20
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 September 1998	24/09/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	M. STANKOVICH ET AL: "C-terminal truncation of the transcriptional activator encoded by areA in Aspergillus nidulans results in both loss-of-function and gain of function phenotypes" MOLECULAR MICROBIOLOGY, vol. 7, no. 1, 1993, pages 81-87, XP002048815	1-15
Y	see the whole document	16-20
X	PLATT A ET AL: "Nitrogen metabolite signalling involves the C-terminus and the GATA domain of the Aspergillus transcription factor AREA and the 3' untranslated region of its mRNA." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 15 (11). 1996. 2791-2801. ISSN: 0261-4189, XP002077390	1-15
Y	see the whole document	16-20
Y	WO 95 35385 A (NOVONORDISK AS ;CHRISTENSEN TOVE (DK); HYNES MICHAEL J (AU)) 28 December 1995 see the whole document especially page 2, paragraph 1	16-20
Y	J. VAN DEN HOMBERGH ET AL: "Aspergillus as a host for heterologous protein production: the problem of proteases" TRENDS IN BIOTECHNOLOGY, vol. 15, July 1997, pages 256-263, XP002048806 CAMBRIDGE GB see page 261, left-hand column, paragraph 3	16-20
Y	DATABASE BIOSIS BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US Accession no 94:532238, 1994 JARAI G AND BUXTON F: "Nitrogen, carbon, and pH regulation of extracellular acidic proteases of Aspergillus niger" XP002077528 see abstract & CURRENT GENETICS, vol. 26, no. 3, 1994, pages 238-244,	16-20

-/--

2

INTERNATIONAL SEARCH REPORT

ernational Application No
PCT/EP 98/02785

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Section Ch, Week 9527 Derwent Publications Ltd., London, GB; Class D13, AN 95-202831 XP002048808 & JP 07 115969 A (ASAHI KASEI KOGYO KK) , 9 May 1995 see abstract	18
Y	EP 0 417 481 A (NESTLE SA) 20 March 1991 cited in the application see the whole document	20

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

ernational Application No
PCT/EP 98/02785

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9535385 A	28-12-1995	AU 2733895 A	15-01-1996
		CN 1150824 A	28-05-1997
		EP 0770139 A	02-05-1997
		FI 965031 A	16-12-1996
		JP 10501414 T	10-02-1998
EP 0417481 A	20-03-1991	CH 679544 A	13-03-1992
		AU 637391 B	27-05-1993
		AU 6106190 A	21-03-1991
		CA 2023474 A,C	13-03-1991
		CN 1050126 A,B	27-03-1991
		DE 69002210 T	21-10-1993
		DK 417481 T	15-11-1993
		ES 2043201 T	16-12-1993
		HK 114294 A	27-10-1994
		JP 2659105 B	30-09-1997
		JP 3112461 A	14-05-1991
		KR 9611710 B	30-08-1996
		MX 172699 B	07-01-1994
		NO 176823 B	27-02-1995
		PT 95276 A,B	22-05-1991
		SG 91694 G	14-10-1994
		US 5141756 A	25-08-1992